

PTO/PCT Rec'd 18 JAN 1998

09/101283

TRANSMITTAL LETTER TO THE UNITED STATES  
DESIGNATED/ELECTED OFFICE (DO/EO/US)  
CONCERNING A FILING UNDER 35 U.S.C. 371

02307E-060010

U.S. APPLICATION NO. (if known, see 37 CFR 1.51)

08/423,409

INTERNATIONAL APPLICATION NO.

PCT/US96/05441

INTERNATIONAL FILING DATE

18 April 1996

PRIORITY DATE CLAIMED

18 April 1995

TITLE OF INVENTION

ALTERED PROTEIN EXPRESSION IN HYPOXIC TROPHOBLASTS

APPLICANT(S) FOR DO/EO/US

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Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. ☒ This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.
2. ☐ This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.
3. ☒ This express request to begin national examination procedures (35 U.S.C. 371(f)) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S.C. 371(b) and PCT Articles 22 and 39(1).
4. ☒ A proper Demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date.
5. ☒ A copy of the International Application as filed (35 U.S.C. 371(c)(2))
  - a. ☒ is transmitted herewith (required only if not transmitted by the International Bureau).
  - b. ☐ has been transmitted by the International Bureau.
  - c. ☐ is not required, as the application was filed in the United States Receiving Office (RO/US)
6. ☐ A translation of the International Application into English (35 U.S.C. 371(c)(2)).
7. ☒ Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371(c)(3))
  - a. ☐ are transmitted herewith (required only if not transmitted by the International Bureau).
  - b. ☐ have been transmitted by the International Bureau.
  - c. ☐ have not been made; however, the time limit for making such amendments has NOT expired.
  - d. ☒ have not been made and will not be made.
8. ☐ A translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).
9. ☒ An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)).
10. ☐ A translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)).

Items 11. to 16. below concern other document(s) or information included:

11. ☐ An Information Disclosure Statement under 37 CFR 1.97 and 1.98.
12. ☐ An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.
13. ☐ A **FIRST** preliminary amendment.  
☐ A **SECOND** or **SUBSEQUENT** preliminary amendment.
14. ☐ A substitute specification.
15. ☐ A change of power of attorney and/or address letter.
16. ☒ Other items or information:  
International Search Report (with references)

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08/423,409

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17. ☒ The following fees are submitted:

Basic National Fee (37 CFR 1.492(a)(1)-(5)):

Search Report has been prepared by the EPO or JPO..... \$930.00

International preliminary examination fee paid to USPTO (37 CFR 1.482)  
..... \$700.00No international preliminary examination fee paid to USPTO (37 CFR 1.482)  
but international search fee paid to USPTO (37 CFR 1.445(a)(2)).. \$770.00Neither international preliminary examination fee (37 CFR 1.482) nor  
international search fee (37 CFR 1.445(a)(2)) paid to USPTO..... \$1,040.00International preliminary examination fee paid to USPTO (37 CFR 1.482)  
and all claims satisfied provisions of PCT Article 33(2)-(4)..... \$96.00

ENTER APPROPRIATE BASIC FEE AMOUNT =

CALCULATIONS | PTO USE ONLY

\$ 930.00

Surcharge of \$130.00 for furnishing the oath or declaration later than ☐ 20 ☒ 30  
months from the earliest claimed priority date (37 CFR 1.492(e)).

\$ 130.00

Claims	Number Filed	Number Extra	Rate
Total claims	22 -20 -	2	X \$22.00
Independent Claims	9 -3 -	6	X \$82.00
Multiple dependent claims(s) (if applicable)			+ \$260.00

\$ 44.00

\$ 492.00

TOTAL OF ABOVE CALCULATIONS =

\$ 1,596

Reduction by 1/2 for filing by small entity, if applicable. Verified Small Entity statement  
must also be filed. (Note 37 CFR 1.9, 1.27, 1.28).

\$

SUBTOTAL =

\$ 1,596

Processing fee of \$130.00 for furnishing the English translation later the ☐ 20 ☐ 30  
months from the earliest claimed priority date (37 CFR 1.492(f)).

+

\$

TOTAL NATIONAL FEE =

\$

Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be  
accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31). \$40.00 per property +

\$

TOTAL FEES ENCLOSED =

\$

Amount to be:  
refunded \$

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- a. ☐ A check in the amount of \$ \_\_\_\_\_ to cover the above fees is enclosed.
- b. ☒ Please charge my Deposit Account No. 20-1430 in the amount of \$ 1,596 to cover the above fees.  
A duplicate copy of this sheet is enclosed.
- c. ☒ The Commissioner is hereby authorized to charge any additional fees associated with this paper or  
during the pendency of this application, or credit any overpayment to the  
Deposit Account No. 20-1430. A duplicate copy of this sheet is enclosed.

NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR  
1.137(a) or (b)) must be filed and granted to restore the application to pending status.

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**ALTERED PROTEIN EXPRESSION IN HYPOXIC**  
**TROPHOBLASTS**

**RELATED APPLICATIONS**

This application is a continuation-in-part of U.S. application Serial No. 08/423,409 filed April 18, 1995 which is incorporated herein by reference for all purposes.

**FIELD OF THE INVENTION**

The present invention relates to the field of reproductive biology. More particularly, the present invention relates to particular proteins whose expression is differentially regulated in hypoxic trophoblasts.

**BACKGROUND OF THE INVENTION**

Implantation and placental development (placentation) involve a complex series of physiological events that result in the physical connection between the mammalian embryo and its mother. Failures in implantation and placental development are clinically important. About one-third of normal human pregnancies end in spontaneous abortion, with 22% of such abortions occurring before pregnancy is even detected clinically (Wilcox *et al.* *N. Engl. J. Med.*, 319: 189 (1988)). The actual abortion rate may be even higher as failures in development during the peri-implantation period account for almost 80% of embryonic loss that occurs in farm animal and, by implication, other species (Roberts, *et al.* *Oxf. Rev. Reprod. Biol.*, 12: 147 (1990), Roberts *et al.*, *Endocr. Rev.*, 13: 432 (1992), King, *Can. Vet. J.*, 32: 99 (1991). Even seemingly minor defects in placentation can have severe negative consequences. In humans, for example, abnormalities in the vascular connections can result in preeclampsia.

Preeclampsia is an idiopathic, life-threatening disease of late pregnancy in which hypertension is associated with hepatic, neurologic, hematologic, or renal involvement. Rapid development of edema, particularly of the ankles, face and hands,

along with a rise in blood pressure, usually signals the onset of this condition. Jaundice and abnormal liver function may be present.

The rapid acceleration of blood pressure elevation is accompanied by an increase in proteinuria, oliguria, edema, and coagulopathy. This is a life threatening syndrome and tends to recur with future pregnancies. At the same time, the baby's normal development slows with accompanying intrauterine growth retardation. In the postpartum period, proteinuric patients are particularly susceptible to the development of postpartum renal failure. Preeclampsia is often characterized by hyperreflexia, visual disturbances and headache indicating neurologic involvement which may ultimately progress to eclampsia characterized by convulsions. Preeclampsia occurs in 7-10% of pregnancies and is responsible for significant maternal and fetal morbidity (Roberts, *Pregnancy-related hypertension.*, pages 703-752 In *Maternal-Fetal Medicine-Principles and Practice*, Creasy & Resnick, eds., W.B. Saunders, Philadelphia (1984).

Once preeclampsia is diagnosed, hospitalization is indicated, since, as described above, the disease can rapidly progress to eclampsia, characterized by convulsions resulting in significant maternal and fetal trauma. The definitive treatment of preeclampsia and eclampsia is delivery of the conceptus, which is carried out promptly, if fetal size and maturity are adequate. If the fetus is immature, management consists of bed rest in a quiet environment and control of neurologic manifestations and blood pressure, the former with magnesium sulfate and the latter usually with vasodilators such as hydralazine and methyldopa. Early detection of preeclampsia and implementation of appropriate therapeutic measures greatly reduces maternal and fetal morbidity. The long term prognosis of promptly detected preeclampsia is generally favorable.

Despite decades of interest and research, the pathogenesis of this disease is still poorly understood. In recent years, however, the availability of the measurement of placental proteins opened new perspectives in the diagnosis of fetoplacental dysfunction. In particular, placental proteins have attracted interest as diagnostic markers of various pathologies during pregnancy. Thus, for example, a decrease in the maternal serum concentration of pregnancy-specific beta-1-glycoprotein (sp<sub>1</sub>) detected by serial measurements could predict fetal malnutrition (Csaba, *Med. Sci.*, 10: 840-842 (1982); Karg *et al. Arch. Gynaekol*, 231: 67-73 (1981).

Biochemical tests of fetoplacental well-being have been applied in the study of preeclampsia (Chard *et al.*, pages 1-93 In: *Placental Function Tests*, Berlin, Springer-Verlag (1982)), sometimes with dramatic clinical significance (Lindberg, *et al. J. Obstet. Gynecol. Br. Commonw.*, 80: 1046-1053 (1973); Spellacy *et al. Am. J. Obstet. Gynecol.*, 109: 588-98 (1971)). However, despite the recent focus on molecular events underlying preeclampsia, relatively little is known regarding the etiology of this and related diseases of pregnancy.

### SUMMARY OF THE INVENTION

The present invention provides an *in vitro* culture system that effectively models abnormal placental function characteristic of an abnormal maternal-placental interface. It was a discovery of the present invention that trophoblast cells cultured under hypoxic conditions attain a morphology, antigenic phenotype, and activity that appears identical to that observed in trophoblasts of an abnormal maternal-placental interface characteristic of various diseases of pregnancy such as threatened abortion, high intrauterine growth retardation, gestational trophoblast diseases including molar pregnancy, choriocarcinoma, placental site tumors, ectopic pregnancy, proteinuria, pregnancy induced hypertension and preeclampsia. In particular, the cells show a decrease in expression levels of  $\alpha 1/\beta 1$  integrins, the 92 kDa type IV collagenase and HLA-G whose upregulation is characteristic of normal trophoblasts, *in vivo*, and trophoblasts cultured identically under normal oxygen conditions.

In addition it was discovered that trophoblasts and explanted chorionic villi cultured under hypoxic conditions alter the expression levels of other, previously unidentified, proteins. These proteins have been isolated and characterized, in terms of molecular weight, pI and whether they are upregulated or down regulated under hypoxic conditions as compared to trophoblasts cultured identically under normal conditions (see Tables 1 and 2). In addition, a number of these proteins have been sequenced and identified in searches of a protein sequence database.

In view of these discoveries, this invention provides for proteins expressed by a mammalian, more preferably a primate or a human, fetal trophoblast cell, or chorionic villus, whose level of expression is substantially altered when the cell, or chorionic villus, is grown under hypoxic conditions. Preferred hypoxic conditions, when referring to *in vitro* culture conditions refer to culture under an atmosphere comprising

less than about 20% oxygen, more preferably less than about 10% oxygen and most preferably less than about 2% oxygen. Hypoxic conditions may include full anoxia (0% oxygen). Preferred hypoxic conditions also include conditions in which the expression levels of  $\alpha 1/\beta 1$ , the 92 kDa type IV collagenase and HLA-G whose, as well as cell  
5 invasiveness, are significantly decreased as compared to trophoblasts cultured identically under normal conditions. Particularly preferred proteins include one or more of the proteins described in Table 1 and Table 2 as having increased or decreased release under hypoxic conditions.

This invention additionally provides for methods of culturing trophoblasts  
10 and/or chorionic villi under hypoxic conditions as described above, and below in the specification. The method may further comprise measuring the expression of a protein selected from the proteins listed in Table 1 and Table 2 as having increased or decreased release under hypoxic conditions.

The proteins expressed by the hypoxic trophoblasts or the hypoxic  
15 chorionic villi of the present invention can be used as markers indicative of the presence of hypoxic conditions which in turn, are indicative of an abnormal maternal-placental interface and consequent abnormal placental function. Thus this invention provides for a method of detecting hypoxic trophoblast cells, or hypoxic chorionic villi, comprising measuring the expression level of a protein whose expression is altered in hypoxic  
20 trophoblasts as compared to normal (normoxic) trophoblasts or whose expression is altered in hypoxic chorionic villi as compared to normal (normoxic) chorionic villi. In a particularly preferred embodiment, the protein is one or more proteins selected from the proteins listed in Tables 1 and 2 as having increased or decreased release under hypoxic conditions. Most preferred proteins include human apolipoprotein A-1 (apo A-1),  
25 placental lactogen, chorionic gonadotropin, and fibrinogen. Preferred detection methods include direct detection of the protein, or alternatively, detection of mRNA encoding the protein. Particularly preferred protein detection methods include immunoassays, while preferred mRNA detection methods include quantitative amplification techniques (*e.g.* quantitative PCR), or hybridization methods such as Northern blots.

30 The present invention also provides a similar assay for detecting abnormal placental function. This involves analyzing a biological sample from a pregnant mammal, more preferably a pregnant primate or human, for abnormal expression of one or more proteins whose expression is altered in a hypoxic trophoblast. Particularly preferred

proteins are one or more proteins listed in Tables 1 and 2 as having increased or decreased release under hypoxic conditions. As indicated above, the hypoxic trophoblast, abnormal placental function, or abnormal maternal-placental interface are indicative or symptomatic of one or more diseases of pregnancy. Such diseases include, but are not limited to threatened abortion, high intrauterine growth retardation, gestational trophoblast diseases including molar pregnancy, choriocarcinoma, placental site tumors, ectopic pregnancy, proteinuria, pregnancy induced hypertension and preeclampsia.

The present invention also provides methods of screening for agents that mitigate the effects of an abnormal maternal-placental interface. These methods involve culturing trophoblasts or chorionic villi under hypoxic conditions (as described above) in the presence of the agent and assaying for changes in the phenotype of the hypoxic trophoblasts, or hypoxic villi, relative to hypoxic trophoblasts or hypoxic chorionic villi cultured without the presence of the agent. The step of assaying for changes in the phenotype may comprise measuring the invasiveness of the trophoblast. Alternatively, the assay may comprise measuring the changes in the levels of expression of one or more proteins expressed (released) by the trophoblasts. Proteins whose expression (release) is typically altered in hypoxic trophoblasts are preferably assayed, while the proteins listed in Tables 1 and 2 as having increased or decreased release under hypoxic conditions, are most preferably assayed.

Conversely, this invention also provides for methods of screening for agents that might induce the formation of an abnormal maternal-placental interface, or abnormal placental function. In this case, the trophoblasts, or chorionic villi, are cultured under normal conditions and assayed for changes in phenotype as compared to normal and/or hypoxic trophoblasts. Phenotypic changes similar to hypoxic trophoblasts or hypoxic chorionic villi are indicative of possible interference or alteration of the maternal-placental interface by the agent. As described above, the assay may comprise measuring the changes in the levels of expression of one or more proteins expressed by the trophoblasts or chorionic villi. Proteins whose expression is typically altered in hypoxic trophoblasts are preferably assayed, while the proteins listed in Tables 1 and 2 as having increased or decreased release under hypoxic conditions are most preferably assayed.

This invention additionally provides a method of modeling, *in vitro*, an abnormal maternal-placental interface or abnormal placental function. The method comprises culturing trophoblast cells in a hypoxic environment as described above.

Finally, this invention provides methods for identifying proteins that are indicative of metastasis and methods for detecting metastatic cells. Methods for identifying proteins indicative of metastasis involve culturing trophoblasts or chorionic villi under hypoxic conditions, as described above; detecting proteins that demonstrate an altered expression level as a result of the hypoxic conditions; and determining if these or related proteins are present in metastatic cells. Preferred proteins are one or more of the proteins listed in Tables 1 and 2 as having increased or decreased release under hypoxic conditions. Preferred methods of determining if the proteins are present involve using antibodies specific for a protein whose expression is altered in hypoxic trophoblasts, more preferably an antibody specific for one of the proteins listed in Tables 1 and 2 as having increased or decreased release under hypoxic conditions.

Methods for detecting metastatic cells involve analyzing a biological sample from a mammal, more preferably a primate or human, for abnormal expression of one or more proteins indicative of metastasis. These preferably include proteins whose expression is altered in a hypoxic trophoblast or hypoxic chorionic villus, or closely related proteins. Particularly preferred proteins are one or more proteins listed in Tables 1 and 2 as having increased or decreased release under hypoxic conditions.

### Definitions

The term "invasiveness", as used herein refers to the ability of a cell to penetrate an extracellular matrix. Methods of measuring invasiveness are well known to those of skill in the art (see, *e.g.*, Librach *et al.* *J. Cell. Biol.*, 113: 437-449 (1991)). Similarly, an invasive cell type or an "invasive cell" refer to a cell capable of penetrating a tissue other than the tissue from which the cell originates. Invasive cells include, but are not limited to trophoblast and malignant cancer cells.

The term "protein", when used herein refers to a chain of amino acids whose  $\alpha$  carbons are linked through peptide bonds. Proteins include native proteins *in vivo* or isolated native proteins. Proteins also include chemically or recombinantly synthesized proteins. In addition, it is to be understood that the term proteins, as used herein includes the protein product as translated from an mRNA molecule as well as the



protein products as subsequently modified. Thus proteins also include modified proteins such as glycoproteins, lipoproteins and the like.

The term "normal oxygen conditions" refers to conditions under which a cells are exposed to the oxygen concentration that they experience *in vivo* in a normal healthy organism. Similarly, the term "normoxic cells" refers to cells exposed to normal oxygen conditions. When trophoblasts are cultured *in vivo* under normal atmospheric oxygen concentrations (about 20%, which, at a standard atmospheric pressure of 760 mm Hg, corresponds to a partial pressure of oxygen ( $pO_2$ ) of about 152 mm Hg) the culture medium has an oxygen concentration of about 13% ( $pO_2 \approx 98$  mm Hg) which corresponds approximately to the oxygen concentration of arterial blood. Thus, the term "normoxic" may be taken herein as referring to trophoblasts subject to an oxygen concentration comparable to trophoblasts cultured under an atmosphere containing a normal oxygen concentration (about 20% or about 152 mm Hg).

As used herein, an "immunoassay" is an assay that utilizes an antibody to specifically bind to the analyte. The immunoassay is characterized by the use of specific binding to a particular antibody as opposed to other physical or chemical properties to isolate, target, and quantify the analyte.

The terms "expression" or "release" of a protein when used herein in reference to a protein whose expression or release is altered under hypoxic conditions are used to indicate that alterations in detectable protein level are due to alterations in the physiological activity of the cell or tissue and not to attribute a particular mechanism to the observed alteration in detectable protein level. Thus the phrases in "increase in expression" or "increase in release" of a protein are used to indicate that some action of the subject cell or tissue results in an increase in the detected levels of that protein, either released to the environment (*e.g.*, culture medium) or detected in a lysate. The increase can be due, for example, to increased expression of a gene encoding that protein, to defective expression of a native protein resulting in the detected protein "fragment", changes in uptake of the protein, changes in active secretion of the protein or changes in net release of the protein.

The phrase "specifically binds to a protein" or "specifically immunoreactive with", when referring to an antibody refers to a binding reaction which is determinative of the presence of the protein in the presence of a heterogeneous population of proteins and other biologics. Thus, under designated immunoassay

conditions, the specified antibodies bind to a particular protein and do not bind in a significant amount to other proteins present in the sample. Specific binding to a protein under such conditions may require an antibody that is selected for its specificity for a particular protein. For example, antibodies can be raised to the trophoblast-secreted (trophoblast-released) proteins (TSPs) of the present invention and not to any other proteins present in a blood sample.

The phrase "a protein of spot n" as used herein, when referring to two dimensional electrophoresis gels describes a protein that occupies the same spatial position, in reference to other protein spots in an electrophoresis gel of the same type of cell or tissue preparation run under the same conditions as the referenced electrophoresis. The letter "n" refers to the spot number. Thus, for example, a protein of spot 7 will appear in a two-dimensional electrophoresis gel in the same position, in relation to the other spots, as the spot identified as spot 7 in Figures 1 and 2.

### BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 and Figure 2 show two-dimensional (PAGE) maps of unlabeled (Coomassie blue-stained) proteins released by chorionic villi grown in F12 HAM/DMEM (1:1/v:v) medium under standard (20% O<sub>2</sub>, Figure 1) and hypoxic (2% O<sub>2</sub>, Figure 2) conditions. Since the gels are 10% polyacrylamide, proteins in the lower molecular weight region of the gel are resolved best. As with cytotrophoblasts, the protein repertoire the cells release under both conditions is remarkably similar. Nevertheless, two types of changes were observed; abundance and pI. A small number of proteins were present in either greater or lesser amounts when the villi were maintained in 2% O<sub>2</sub>. Protein abundance changes are characterized by the following symbols: ○ down in hypoxia; □ up in hypoxia; and Δ unchanged. Changes in apparent pI are attributed to changes in posttranslational modifications such as glycosylation and/or phosphorylation.

### DETAILED DESCRIPTION

#### I. Hypoxic Trophoblasts or Hypoxic Chorionic Villi as Models of Abnormal Maternal-Placental Interface.

This invention involves the discovery that trophoblasts and/or chorionic villi grown under hypoxic conditions provide a useful *in vitro* model of the abnormal

maternal-placental interface and various disease states that are characteristic of, or result from, abnormal placental-maternal interaction or function. In particular, it is a discovery of the present invention that hypoxic trophoblasts express a gross morphology, a histology, an antigenic phenotype and a loss of invasiveness that is identical to that found in cells characteristic of the abnormal maternal-placental interface characteristic of a number of diseases of pregnancy. Such diseases include, but are not limited to, threatened abortion, high intrauterine growth retardation, gestational trophoblast diseases including molar pregnancy, choriocarcinoma, placental site tumors, ectopic pregnancy, proteinuria, pregnancy induced hypertension and preeclampsia. The *in vitro* models of the present invention thus provides an excellent system in which to screen for therapeutic agents useful in the treatment of various diseases of pregnancy involving an abnormal maternal-placental interface.

In addition, the present invention provides for a number of proteins whose expression level is dramatically altered (either upregulated or downregulated) in hypoxic trophoblasts and hypoxic chorionic villi. These proteins provide useful markers for the early diagnosis of abnormal placental function and thus of diseases, such as preeclampsia, characteristic of abnormal placental function. As it is believed that these proteins are responsible for various complications of pregnancy including (1) an alteration in vascular reactivity associated with a hypersensitivity to infused angiotensin; (2) a decrease in production of prostacyclin with an associated increase in production of thromboxanes; (3) a decrease in renal hemodynamics, due at least in part to glomerular endotheliosis; (4) a widespread endothelial disorder, resulting in the loss of albumin from the intravascular space; and (5) an inherent immunologic misadaptation during placentation, resulting in incomplete trophoblast invasion of the spiral arterioles pathophysiological abnormalities. Detection of expression levels of these proteins provides a screening system for possible therapeutic agents that might mitigate these and other adverse effects of abnormal placental differentiation.

Finally, because of the similarity between trophoblasts and other invasive cell types (*e.g.* metastatic cells), the *in vitro* model of the present invention provides an excellent system for the identification of proteins that mediate cellular invasion of a tissue and for the screening of therapeutic agents that may inhibit the invasive activity of such cells.

**A) The abnormal maternal-placental interface**

The structure and function of the maternal-placental interface is mediated by the action of trophoblast cells. Trophoblasts are specialized epithelial cells of the placenta that physically connect the embryo and the uterus. In primates and rodents, trophoblasts are invasive, breaching uterine blood vessels and thereby achieving direct contact with maternal blood. In particular, human trophoblasts are extremely invasive; they traverse the uterine epithelium and invade the decidua, the inner third of the myometrium and the maternal arteries.

As used herein, the term "trophoblasts" includes the cytotrophoblast stem cells and lineages derived from these stem cells. The various lineages derived from cytotrophoblast stem cells are generally known to those of skill in the art. In humans, for example, two differentiation pathways exist for cytotrophoblasts, giving rise to populations that are morphologically and functionally distinct (Cross *et al. Science*, 266: 1508-1518 (1994)). In the first trimester, cytotrophoblast stem cells reside in chorionic villi of two types; "floating" villi that do not contact the uterine wall and "anchoring" villi that do contact the uterine wall. Cytotrophoblasts in the floating villi exist only as polarized epithelial monolayers, anchored to a basement membrane and surrounding a stromal core containing fetal blood vessels. These cytotrophoblasts, which are highly proliferative in the first trimester of gestation, differentiate exclusively by fusing to form a syncytial layer that covers the villus. Floating villi, which make up the fetal compartment of the placenta, are bathed by maternal blood and perform gas and nutrient exchange functions.

In contrast, anchoring villi contain cytotrophoblast stem cells that enter both differentiation pathways. In much of the anchoring villus, cytotrophoblasts fuse to form a syncytium. However, at selected sites, cytotrophoblasts break through the syncytium and form multilayered columns of nonpolarized cells. Anchoring villi physically connect the embryo to the uterine wall via these cell columns, and give rise to the most highly invasive and migratory cytotrophoblasts. These invasive trophoblasts (also known as intermediate trophoblasts, cytotrophoblasts, or x-cells) invade uterine blood vessels.

**B) *In vitro* model of the abnormal maternal-placental interface.**

A large body of evidence indicates that preeclampsia, and other diseases of pregnancy, are associated with highly characteristic abnormalities in placental development (referred to herein has an "abnormal maternal-placental interface") such that the placenta is only superficially connected to the uterus. Cytotrophoblast invasion is shallow and does not proceed beyond the decidual portions of the spiral arteries.

(Redman, *New Engl. J. Med.* 323: 478 (1990); Brosens *et al. Obster, Gynecol. Annu.* 1: 177 (1972) Gerretsen *et al., Brit. J. Obstet. Gynecol.*, 88: 876 (1981); Moodley and Ramsaroop, *S. Afr. Med. J.*, 75: 376 (1989)). In addition, not as many vessels show evidence of trophoblast invasion (Khong *et al. Br. J. Obstet. Gynecol.*, 93: 1049 (1986)).

These morphological differences are a dramatic contrast to normal development (placental differentiation) in which, as explained above, the trophoblasts, detach from their basement membranae, aggregate, and invade much of the uterus and its arterial system thereby forming an intimate connection (the maternal-placental interface) between the mother and the fetus. As used herein, the term "abnormal placental function" refers to the physiological consequences of this abnormal placental development.

It was a discovery of the present invention that hypoxia plays a role in this abnormal development process leading to superficial connection between the placenta and the uterus. Without being bound to a particular theory, it is believed that the lack of uterine blood vessel invasion by trophoblasts, which typically initiates maternal blood flow to the placenta in normal pregnancy, makes this tissue relatively hypoxic under preeclamptic conditions. The resulting hypoxia is associated with the differential regulation of a number of proteins that are released to the maternal circulation.

It was a discovery of the present invention that cytotrophoblasts cultured under hypoxic conditions (as described in Examples 1 and 2) showed abnormal differentiation that was *identical* to that observed in preeclampsia *in vivo*. The cells expressed the *same* altered pattern of stage-specific antigens as that seen *in vivo*. In particular, the hypoxic cells expressed very low levels of  $\alpha 1/\beta 1$ , the 92 kDa type IV collagenase and HLA-G whose upregulation is characteristic of normal cytotrophoblasts. Also as in preeclampsia, the cells' invasiveness was greatly reduced. This is the exact same antigenic phenotype that characterizes cytotrophoblast differentiation in preeclampsia and indicates that these hypoxic trophoblast cultures can be used to identify

unique proteins characteristic of an abnormal maternal-placental interface. Such proteins are candidates for the toxic placental factors that are thought to produce the maternal syndrome associated with preeclampsia and other diseases of pregnancy.

Similarly, it was also a discovery of this invention that chorionic villi (e.g., anchoring chorionic villi) cultured under hypoxic conditions also showed altered release of proteins that can be used as markers of an abnormal maternal-placental interface. In particular, these proteins appear to be good markers of preeclampsia and other diseases of pregnancy.

Thus, in one embodiment, this invention provides for an *in vitro* model of abnormal maternal-placental interface or abnormal placental function. This model comprises culturing cytotrophoblasts and/or chorionic villi under hypoxic conditions. As used herein, hypoxic conditions are culture conditions under which the trophoblasts or chorionic villi are exposed to oxygen concentrations less than those to which they are exposed *in vivo* in a normal healthy pregnancy. When trophoblasts or chorionic villi are cultured under normal atmospheric oxygen concentrations (about 20% O<sub>2</sub>) the culture medium contains about 13% oxygen (pO<sub>2</sub> ≈ 98 mm Hg) which is comparable the physiological oxygen concentrations trophoblasts experience *in vivo* in a normal, healthy pregnancy. Thus, hypoxic conditions refers to culture conditions in which cells are grown under an atmosphere containing less than about 20% oxygen, more preferably less than about 10% oxygen and most preferably less than about 2% oxygen. As trophoblasts are capable of growing when fully anoxic, as used herein, hypoxic conditions include fully anoxic conditions.

The trophoblasts may be isolated and cultured according to any of a number of means well known to those of skill in the art. In a preferred embodiment, the trophoblast cells are isolated and cultured according to the methods described by Fisher *et al. J. Cell. Biol.*, 109: 891-902 (1989) and Librach *et al., J. Cell. Biol.*, 113: 437-449 (1991) and detailed in Example 1. Generally, first and second trimester placentas are obtained immediately after vacuum aspiration, and the chorionic villi are prepared as described by Fisher *et al. J. Cell. Biochem.*, 27: 31-41 (1985). The isolation of cytotrophoblast cells from villi is performed as described in Fisher *et al., Troph. Res.*, 4: 115-138 (1990). Briefly, the washed villus pellet is incubated for either 20 (first trimester) or 30 min (second trimester) (5:1 vol/wt/wt) in enzymatic dissociation solution I (PBS containing 500 U/ml collagenase [type IV; Sigma Chemical Co., St. Louis,

Missouri, USA], 200 U/ml hyaluronidase [type 1-S; Sigma Chemical Co.], 0.2 mg/ml Dnase [type IV; Sigma Chemical Co.], and 1 mg/ml BSA). The villi are separated from the supernatant, which contains the syncytium, by centrifugation and incubated for 10 minutes in dissociation solution II (PBS containing 0.25% trypsin [type XII; Sigma Chemical Co.], 2mM EDTA, and 0.2 mg/ml DNase). The dissociated cells are isolated by centrifugation, resuspended in 4 ml of medium containing 10% FCS and layered over a preformed Percoll gradient made up in Hanks' balanced salt solution according to the method of Kliman *et al.*, *Endocrinol.*, 118: 1567-1582 (1986). The gradient is centrifuged (1,000 x g) for 25 min at room temperature, after which a broad band in the middle of the tube containing the cytotrophoblast cells is removed. The cells are washed several times and resuspended in MEM D-valine medium (Gilbert and Migeon, *Cell*, 5: 11-17 (1975)) containing either 20% dialyzed FCS or 2% Nutridoma, 1% glutamine, and 50 µg/ml gentamicin.

Then 1 ml, containing about  $5 \times 10^5$  cytotrophoblasts, is added per 15-mm tissue culture well. In most cases, either the wells or coverslips (12 mm diameter) placed within the wells, are coated with an extracellular matrix (ECM) produced by PF HR9 cells as described by Fisher *et al.* *J. Cell. Biochem.*, 25: 31-41 (1985). Under these conditions, the cells adhere to produce a confluent monolayer within 4 hours.

To isolate term cytotrophoblasts, the washed villus pellet is subjected to three cycles of trypsinization (dissociation solution II) and the resulting cells are purified on a Percoll gradient as described above.

Similarly, chorionic villi can be isolated and cultured according to standard methods known to those of skill in the art. For example, human chorionic villi can be dissected from placentas (*e.g.*, first trimester placentas) and cultured in F12 HAM/DMEM (1:1/v:v) culture medium. Detailed culture methods are provided in Example 4.

One of skill will appreciate that other compositions may be added to the culture medium. These may include, but are not limited to, various labeled amino acids (*e.g.* radio-labeled amino acids such as [ $^{35}$ S]cysteine and [ $^{35}$ S]methionine, [ $^3$ H]leucine and the like) to facilitate the quantitative analysis of newly synthesized proteins, or various pharmacological agents to be tested for their effect on cytotrophoblast differentiation.

One of skill will appreciate that there are numerous variations of this isolation and culture procedure that may be utilized. However, where it is desired to

isolate proteins expressed by the cultured trophoblasts, it is preferred to use culture medium lacking serum.

## **II. Proteins Characteristic of Abnormal Placental Function.**

5 It was also a discovery of the present invention that hypoxic trophoblasts and hypoxic chorionic villi express various proteins at altered levels as compared to the identical cells cultured under normoxic conditions. These proteins appear to be characteristic of hypoxic trophoblasts and/or hypoxic chorionic villi. Thus, in one embodiment, this invention provides for proteins expressed by mammalian fetal  
10 trophoblast cells and/or chorionic villi grown under hypoxic conditions as described above. In particular, this invention provides for proteins that are over or under-expressed as much as five-fold as compared to identical cells under normoxic conditions.

Proteins from hypoxic trophoblasts were isolated using 2-dimensional electrophoresis (2D SDS-PAGE) as according to the method of Patton *et al.*

15 *Biotechniques* 8: 518(1990), with precautions suggested by Hunkapillar *et al. Methods in Enzymology*, 91: 227 (1983) as described in Example 2. After calculation of molecular weight from the PAGE gel, the isoelectric point (pI) was determined by isoelectric focussing. Hypoxic trophoblast proteins are listed in Table 1, which includes molecular weights, isoelectric points and whether the protein is upregulated or down regulated in  
20 hypoxic trophoblasts. Proteins identified from hypoxic chorionic villi are identified in Table 2 (*See, example 3*).



**Table 1.** Molecular weight and pI of proteins whose expression is altered in hypoxic trophoblasts.

Protein	Molecular Weight* (kDa)	pI	Expression
A	21	6.0	increased
B	22	7.0	increased
C	23	7.5	increased
D	55	8.5	increased
E	62	5.5	increased
F	40	4.5	decreased
G	67	6.5	decreased
H	75	9.0	decreased

\* Molecular weights are estimated from electrophoretic gels ( $\pm 5$  kDa).

The 2D gels gave no evidence of overlapping spots indicating that each of these proteins was fully isolated. One of skill in the art, will appreciate that the proteins may be further characterized by a number of means including, but not limited to, amino acid analysis and sequencing. Methods of further purification, amino acid analysis and sequencing are routine and well known in the art. These include, but are not limited to protein purification methods as described in *Methods in Enzymology, Guide to Protein Purification*, M. Deutscher, ed. Vol. 182 (1990), as well as various cloning and sequencing strategies as described by Sambrook, *et al.*, *Molecular Cloning: A Laboratory Manual 2nd Ed.*, Vols. 1-3, Cold Spring Harbor Laboratory (1989); *Methods in Enzymology, Vol. 152: Guide to Molecular Cloning Techniques*, Berger and Kimmel, eds., San Diego: Academic Press, Inc. (1987)), or *Current Protocols in Molecular Biology*, Ausubel, *et al.*, eds., Greene Publishing and Wiley-Interscience, New York (1987).

In a particularly preferred embodiment, the proteins are initially isolated using a preparative 2D gel. The isolated proteins are then sequenced using mass spectrometry methods as detailed in Example 3.

Of course, the proteins listed in Table 1, are not the only proteins whose expression is altered in hypoxic cytotrophoblasts or hypoxic chorionic villi. The present invention thus provides for a method of identifying proteins whose expression is altered under hypoxic conditions. This method includes the steps of culturing cells, under

hypoxic conditions and detecting proteins whose expression is altered. In a preferred embodiment, the cells are trophoblast cells or cells in explanted chorionic villi, cultured as described above. The proteins whose expression is altered are preferably identified by 2D SDS-PAGE as described above and in Example 2.

### III. Screening for an Abnormal Placental-Maternal Interface.

The proteins whose expression is altered in hypoxic trophoblasts (referred to herein as trophoblast-secreted or trophoblast-released proteins or TSPs) or in hypoxic chorionic villi (referred to as chorionic villus-released proteins), more preferably the proteins listed in Table 1, may be used as markers of abnormal placental function (*e.g.* as caused by an abnormal maternal-placental interface) and therefore as markers for the resulting diseases of pregnancy, such as preeclampsia. This invention thus provides for methods of detecting an abnormal placental-maternal interface, hypoxic trophoblasts, hypoxic chorionic villi, abnormal placental function, or a disease of pregnancy (*e.g.* preeclampsia) by detecting or measuring the expression levels of a protein whose expression level is altered in hypoxic trophoblasts or chorionic villi as compared to normoxic trophoblasts or normal chorionic villi, respectively. In a preferred embodiment, the method involves determining the expression level of one or more of the proteins listed in Table 1.

While the expression levels of the protein may be determined *in vivo*, in a preferred embodiment, they are determined *ex vivo* in a biological sample or in a culture derived from a biological sample. The proteins are preferably quantified in a biological sample derived from a patient. As used herein, a biological sample is a sample of biological tissue or fluid that contains a concentration of the protein being screened that may be correlated with *in vivo* trophoblast expression levels of the same protein. Particularly preferred biological samples include blood, chorion villus biopsy, amniocentesis and cervicovaginal secretions. See, for example, U.S. Patent No. 5,096,830, incorporated herein by reference, which describes cervicovaginal secretions as diagnostic assay samples, and provides means for taking such samples.

In another preferred embodiment, trophoblast-secreted proteins (TSPs) are quantified in whole blood or blood derivatives such as blood plasma or blood serum. Blood samples are isolated from a patient according to standard methods well known to those of skill in the art, most typically by venipuncture. Although the sample is typically

taken from a human patient, the assays can be used to detect trophoblast-secreted proteins in samples from any mammal, such as primates, rodents, canines, felines, bovines and porcines.

The sample may be pretreated as necessary by dilution in an appropriate buffer solution or concentrated, if desired. Any of a number of standard aqueous buffer solutions, employing one of a variety of buffers, such as phosphate, Tris, or the like, at physiological pH can be used.

In a preferred embodiment, assays are performed using blood plasma (blood lacking a cellular component) or blood serum (blood lacking a cellular component and clotting factors). Means of preparing blood plasma are well known to those of skill in the art and typically involve centrifugation or filtration to produce blood plasma, or clotting followed by centrifugation or filtration to produce blood serum. The blood plasma or serum may be diluted by the addition of buffers or other reagents well known to those of skill in the art and may be stored for up to 24 hours at 2-8°C, or at -20°C or lower for longer periods, prior to measurement of TSPs.

#### **A) Quantification of trophoblast-secreted proteins.**

The trophoblast-secreted proteins (TSPs) and proteins released by the chorionic villi of this invention may be detected and quantified by any of a number of means well known to those of skill in the art. These may include analytic biochemical methods such as electrophoresis, capillary electrophoresis, high performance liquid chromatography (HPLC), thin layer chromatography (TLC), hyperdiffusion chromatography, 2D electrophoresis as described in Example 2, and the like, or various immunological methods such as fluid or gel precipitin reactions, immunodiffusion (single or double), immunoelectrophoresis, radioimmunoassay(RIA), enzyme-linked immunosorbent assays (ELISAs), immunofluorescent assays, western blotting, and the like.

As used herein, an immunoassay is an assay that utilizes an antibody to specifically bind to the analyte. The immunoassay is characterized by the use of specific binding of the trophoblast secreted protein to a particular antibody as opposed to other physical or chemical properties to isolate, target, and quantify the analyte.

##### **1) Antibodies to trophoblast- or chorionic villus-released proteins.**

Antibodies can be raised to the trophoblast- or chorionic villus-released proteins of the present invention using routine methods well known to those of skill in the art. As used herein "antibodies" include immunoglobulin or a population of immunoglobins which specifically bind to an antigen. Thus an antibody may be  
5 monoclonal or polyclonal including individual, allelic, strain, or species variants, both in their naturally occurring (full-length) forms, various antibody fragmentary forms and in recombinant forms. Additionally antibodies is intended to refer to recombinantly expressed antibodies as in phage-display libraries. Antibodies can be raised to the trophoblast- or chorionic villus-released proteins in either their native configurations or in  
10 non-native configurations. Anti-idiotypic antibodies may also be used.

The isolated cytotrophoblast/chorionic villus proteins of the present invention, more preferably the proteins listed in Table 1, may be used as antigens to raise trophoblast or chorionic villus protein specific antibodies. In brief, an immunogen, (the purified trophoblast- or chorionic villus-released protein) is mixed with an adjuvant and animals are immunized with the mixture. The specific amounts will vary in  
15 accordance with the protein and the animals used. In general, 1 to 2 mg/kg of body weight are injected and about 1 to 3 doses is common. The animal's immune response to the immunogen preparation is monitored by taking test bleeds and determining the titer of reactivity to the polypeptide of interest. When appropriately high titers of antibody to the immunogen are obtained, blood is collected from the animal and antisera are  
20 prepared. Further fractionation of the antisera to enrich for antibodies reactive to the polypeptide is performed where desired. See, e.g., Coligan (1991) *Current Protocols in Immunology* Wiley/Greene, NY; and Harlow and Lane (1989) *Antibodies: A Laboratory Manual* Cold Spring Harbor Press, NY.

25 Monoclonal antibodies may be obtained by various techniques familiar to those skilled in the art. Description of techniques for preparing such monoclonal antibodies may be found in, e.g., Stites *et al.* (eds.) *Basic and Clinical Immunology* (4th ed.) Lange Medical Publications, Los Altos, CA, and references cited therein; Harlow and Lane (1988) *Antibodies: A Laboratory Manual* CSH Press; Goding (1986)  
30 *Monoclonal Antibodies: Principles and Practice* (2d ed.) Academic Press, New York, NY; and particularly in Kohler and Milstein (1975) *Nature* 256: 495-497, which discusses one method of generating monoclonal antibodies.

Summarized briefly, this method involves injecting an animal with an immunogen. The animal is then sacrificed and cells taken from its spleen, which are then fused with myeloma cells (*See, Kohler and Milstein (1976) Eur. J. Immunol. 6: 511-519, incorporated herein by reference*). The result is a hybrid cell or "hybridoma" that is capable of reproducing *in vitro*.

Colonies arising from single immortalized cells are screened for production of antibodies of the desired specificity and affinity for the antigen, and yield of the monoclonal antibodies produced by such cells is enhanced by various techniques, including injection into the peritoneal cavity of a vertebrate host. Alternatively, one may isolate DNA sequences which encode a monoclonal antibody or a binding fragment thereof by screening a DNA library from human B cells according to the general protocol outlined by Huse *et al.* (1989) *Science* 246: 1275-1281. In this manner, the individual antibody species obtained are the products of immortalized and cloned single B cells from the immune animal generated in response to a specific site recognized on the immunogenic substance.

## **2) Immunological Binding Assays.**

A particular trophoblast- or chorionic villus-released protein can be measured by a variety of immunoassay methods. For a review of immunological and immunoassay procedures in general, see *Basic and Clinical Immunology* (7th ed.), Stites and Terr, eds. (1991). Moreover, the immunoassays of the present invention can be performed in any of several configurations (see, *e.g.*, those reviewed in *Enzyme Immunoassay*, Maggio ed. CRC Press, Boca Raton, Florida (1980); Tijan, *Practice and Theory of Enzyme Immunoassays, Laboratory Techniques in Biochemistry and Molecular Biology*, Elsevier Science Publishers, Amsterdam (1985); and Harlow and Lane *Antibodies, A Laboratory Manual, supra. Immunoassay: A Practical Guide* Academic Press, Chan, ed. Orlando, FL (1987); *Principles and Practice of Immunoassays*, Price and Newman, eds. Stockton Press, N.Y. (1991); and *Non-isotopic Immunoassays*, Ngo ed. Plenum Press, N.Y. (1988); *Methods in Cell Biology Volume 37: Antibodies in Cell Biology*, Asai, ed. Academic Press, Inc. New York (1993); and U.S. Patents 4,366,241; 4,376,110; 4,517,288; and 4,837,168).

Immunological binding assays (or immunoassays) typically utilize a "capture agent" to specifically bind to and often immobilize the analyte (in this case

trophoblast- or chorionic villus-released proteins). The capture agent is a moiety that specifically binds to the analyte. In a preferred embodiment, the capture agent is an antibody that specifically binds the trophoblast-secreted proteins listed in Table 1.

Immunoassays also often utilize a labeling agent to specifically bind to and label the binding complex formed by the capture agent and the analyte. The labeling agent may itself be one of the moieties comprising the antibody/analyte complex. Thus, the labeling agent may be a labeled trophoblast- or chorionic villus-released protein or a labeled anti-trophoblast- or chorionic villus-released protein antibody. Alternatively, the labeling agent may be a third moiety, such as another antibody, that specifically binds to the antibody/trophoblast- or chorionic villus-released protein complex.

In a preferred embodiment, the labeling agent is an antibody that specifically binds to the capture agent (*e.g.*, anti-trophoblast-released protein antibody). Such agents are well known to those of skill in the art, and most typically comprise labeled antibodies that specifically bind antibodies of the particular animal species from which the capture agent is derived. Thus, for example, where the capture agent is a mouse derived anti-human Trophoblast-secreted proteins antibody, the label agent may be a goat anti-mouse IgG; an antibody that is specific to the constant region of the mouse antibody.

Other proteins capable of specifically binding immunoglobulin constant regions, such as protein A or protein G may also be used as the label agent. These proteins are normal constituents of the cell walls of streptococcal bacteria. They exhibit a strong non-immunogenic reactivity with immunoglobulin constant regions from a variety of species. See, generally Kronval, *et al.*, *J. Immunol.*, 111:1401-1406 (1973), and Akerstrom, *et al.*, *J. Immunol.*, 135:2589-2542 (1985).

Throughout the assays, incubation and/or washing steps may be required after each combination of reagents. Incubation steps can vary from about 5 seconds to several hours, preferably from about 5 minutes to about 24 hours. However, the incubation time will depend upon the assay format, analyte, volume of solution, concentrations, and the like. Usually, the assays will be carried out at ambient temperature, although they can be conducted over a range of temperatures, such as 10°C to 40°C.

**a. Non-competitive assay formats.**

Immunoassays for detecting the trophoblast- or chorionic villus-released proteins of the present invention may be either competitive or noncompetitive. Noncompetitive immunoassays are assays in which the amount of captured analyte (in this case a trophoblast-secreted protein) is directly measured. In one preferred "sandwich" assay, for example, the capture agent (*e.g.*, anti-trophoblast protein antibodies) can be bound directly to a solid substrate where they are immobilized. These immobilized antibodies then capture a trophoblast-released or chorionic villus-released protein present in the test sample. The protein thus immobilized is then bound by a labeling agent, such as a second human anti-trophoblast-released protein antibody bearing a label. Alternatively, the second antibody may lack a label, but it may, in turn, be bound by a labeled third antibody specific to antibodies of the species from which the second antibody is derived.

**b. Competitive assays.**

In competitive assays, the amount of analyte (trophoblast-released protein or chorionic villus-released protein) present in the sample is measured indirectly by measuring the amount of an added (exogenous) analyte (*e.g.*, trophoblast-secreted protein) displaced (or competed away) from a capture agent (*e.g.*, anti-trophoblast protein antibody) by the analyte present in the sample. In one competitive assay, a known amount of, in this case, trophoblast-released or chorionic villus-released protein is added to the sample and the sample is then contacted with a capture agent, in this case an antibody that specifically binds the trophoblast-released or chorionic villus-released protein. The amount of trophoblast-released or chorionic villus-released protein bound to the antibody is inversely proportional to the concentration of trophoblast-released or chorionic villus-released protein present in the sample.

In a particularly preferred embodiment, the antibody is immobilized on a solid substrate. The amount of analyte (*e.g.*, trophoblast-secreted protein) bound to the antibody may be determined either by measuring the amount of analyte present in an analyte protein/antibody complex, or alternatively by measuring the amount of remaining uncomplexed analyte protein. The amount of analyte protein may be detected by providing a labeled analyte proteins.

A hapten inhibition assay is another preferred competitive assay. In this assay a known analyte (*e.g.*, a trophoblast-secreted protein) is immobilized on a solid substrate. A known amount of anti-analyte antibody (*e.g.*, anti-trophoblast-secreted protein antibody) is added to the sample, and the sample is then contacted with the immobilized analyte protein. In this case, the amount of anti-analyte antibody bound to the immobilized analyte protein is inversely proportional to the amount of analyte present in the sample. Again the amount of immobilized antibody may be detected by detecting either the immobilized fraction of antibody or the fraction of the antibody that remains in solution. Detection may be direct where the antibody is labeled or indirect by the subsequent addition of a labeled moiety that specifically binds to the antibody as described above.

#### **c. Reduction of non-specific binding.**

One of skill in the art will appreciate that it is often desirable to reduce non-specific binding in immunoassays. Particularly, where the assay involves an antigen or antibody immobilized on a solid substrate it is desirable to minimize the amount of non-specific binding to the substrate. Means of reducing such non-specific binding are well known to those of skill in the art. Typically, this involves coating the substrate with a proteinaceous composition. In particular, protein compositions such as bovine serum albumin (BSA), nonfat powdered milk, and gelatin are widely used with powdered milk being most preferred.

#### **3) Other assay formats.**

Western blot (immunoblot) analysis can also be used to detect and quantify the presence of trophoblast-released or chorionic villus-released proteins in the sample. The technique generally comprises separating sample proteins by gel electrophoresis on the basis of molecular weight, transferring the separated proteins to a suitable solid support, (such as a nitrocellulose filter, a nylon filter, or derivatized nylon filter), and incubating the sample with the antibodies that specifically bind trophoblast-released or chorionic villus-released proteins. The anti-analyte antibodies specifically bind to analyte proteins on the solid support. These antibodies may be directly labeled or alternatively may be subsequently detected using labeled antibodies (*e.g.* labeled sheep anti-mouse



antibodies) that specifically bind to the anti-trophoblast-released or anti-chorionic villus-released protein antibodies.

Other assay formats include liposome immunoassays (LIA), which use liposomes designed to bind specific molecules (*e.g.*, antibodies) and release encapsulated reagents or markers. The released chemicals are then detected according to standard techniques (see, Monroe *et al.*, *Amer. Clin. Prod. Rev.* 5:34-41 (1986)).

### **B) Detection of Nucleic Acids Encoding Trophoblast-Released or Chorionic Villus-Released Proteins.**

Changes in the expression levels of trophoblast-released and/or chorionic villus-released proteins may also be detected by measuring changes in the amount of transcribed mRNA that encodes the trophoblast-released and/or chorionic villus released proteins. Means of detecting mRNA levels are well known to those of skill in the art. Preferred methods include hybridization and amplification methods.

#### **1) Hybridization methods.**

Similarly, a Northern transfer (Northern blot) may be used for the quantification of mRNA encoding trophoblast-released or chorionic villus-released proteins. In brief, the total nucleic acid is isolated from a given cell sample using, for example, an acid guanidinium-phenol-chloroform extraction method and mRNA is isolated by oligo dT column chromatography. The mRNA is then electrophoresed in a suitable electrophoretic gel to separate the mRNA species and the mRNA is transferred from the gel to a nitrocellulose membrane. As with the Southern blots, labeled probes are used to identify the presence or absence of mRNA encoding trophoblast-secreted and/or chorionic villus-released proteins.

A variety of nucleic acid hybridization formats are known to those skilled in the art. For example, common formats include sandwich assays and competition or displacement assays. Hybridization techniques are generally described in *Nucleic Acid Hybridization, A Practical Approach*, Ed. Hames, B.D. and Higgins, S.J., IRL Press, (1985); Gall and Pardue, *Proc. Natl. Acad. Sci., U.S.A.*, 63: 378-383 (1969); and John *et al.*, *Nature*, 223: 582-587 (1969).

For example, sandwich assays are commercially useful hybridization assays for detecting or isolating nucleic acid sequences. Such assays utilize a "capture" nucleic acid covalently immobilized to a solid support and a labeled "signal" nucleic acid

in solution. The clinical sample will provide the target nucleic acid. The "capture" nucleic acid and "signal" nucleic acid probe hybridize with the target nucleic acid to form a "sandwich" hybridization complex. To be effective, the signal nucleic acid cannot hybridize with the capture nucleic acid.

5 Typically, labelled signal nucleic acids are used to detect hybridization. Complementary nucleic acids or signal nucleic acids may be labelled by any one of several methods typically used to detect the presence of hybridized polynucleotides. The most common method of detection is the use of autoradiography with  $^3\text{H}$ ,  $^{125}\text{I}$ ,  $^{35}\text{S}$ ,  $^{14}\text{C}$ , or  $^{32}\text{P}$ -labelled probes or the like. Other labels include ligands which bind to labelled  
10 antibodies, fluorophores, chemiluminescent agents, enzymes, and antibodies which can serve as a specific binding pair member for a labeled ligand. Labels are discussed in more detail below.

Detection of a hybridization complex may require the binding of a signal generating complex to a duplex of target and probe polynucleotides or nucleic acids. Typically, such binding occurs through ligand and anti-ligand interactions as between a  
15 ligand-conjugated probe and an anti-ligand conjugated with a signal.

The label may also allow indirect detection of the hybridization complex. For example, where the label is a hapten or antigen, the sample can be detected by using antibodies. In these systems, a signal is generated by attaching fluorescent or enzyme molecules to the antibodies or, in some cases, by attachment to a radioactive label. (see, *e.g.*, pp 9-20 in *Practice and Theory of Enzyme Immunoassays*, Laboratory Techniques  
20 in Biochemistry and Molecular Biology, Burdon, R.H., van Knippenberg, P.H., Eds., Elsevier (1985))

The sensitivity of the hybridization assays may be enhanced through use  
25 of a nucleic acid amplification system which multiplies the target nucleic acid being detected. Examples of such systems include the polymerase chain reaction (PCR) system and the ligase chain reaction (LCR) system. Other methods recently described in the art are the nucleic acid sequence based amplification (NASBA, Cingone, Mississauga, Ontario) and Q Beta Replicase systems.

30 An alternative means for determining the level of expression of a gene encoding a trophoblast secreted protein is *in situ* hybridization. *In situ* hybridization assays are well known and are generally described in Angerer, *et al.*, *Methods Enzymol.*, 152: 649-660 (1987). In an *in situ* hybridization assay, cells or tissue

specimens are fixed to a solid support, typically a glass slide. If DNA is to be probed, the cells are denatured with heat or alkali. The cells are then contacted with a hybridization solution at a moderate temperature to permit annealing of labeled probes specific to nucleic acids encoding trophoblast-released and/or chorionic villus-released proteins. The probes are preferably labelled with radioisotopes or fluorescent reporters.

## **2) Amplification methods.**

Transcription levels of mRNA may also be quantified by nucleic acid amplification methods such as quantitative polymerase chain reaction (quantitative PCR). Typically quantitative PCR involves simultaneously co-amplifying a known quantity of a control sequence using the same primers. This provides an internal standard that may be used to calibrate the PCR reaction.

One preferred internal standard is a synthetic AW106 cRNA. The AW106 cRNA is combined with RNA is isolated from the sample according to standard techniques known to those of skill in the art. The RNA is then reverse transcribed using a reverse transcriptase to provide copy DNA. The cDNA sequences are then amplified using labeled primers. The amplification products are separated, typically by electrophoresis, and the amount of radioactivity (proportional to the amount of amplified product) is determined. The amount of mRNA in the sample is then calculated by comparison with the signal produced by the known AW106 RNA standard. Detailed protocols for quantitative PCR are provided in *PCR Protocols, A Guide to Methods and Applications*, Innis *et al.*, Academic Press, Inc. N.Y., (1990).

## **C) Labels.**

The particular label or detectable group used in the assay is not a critical aspect of the invention, so long as it does not significantly interfere with the specific binding of the antibody or nucleic acid used in the assay. The detectable group can be any material having a detectable physical or chemical property. Such detectable labels have been well-developed in the field of immunoassays nucleic acid hybridization and, in general, most any label useful in such methods can be applied to the present invention. Thus, a label is any composition detectable by spectroscopic, photochemical, biochemical, immunochemical, electrical, optical or chemical means. Useful labels in the present invention include magnetic beads (*e.g.* Dynabeads™), fluorescent dyes (*e.g.*,

fluorescein isothiocyanate, texas red, rhodamine, and the like), radiolabels (*e.g.*,  $^3\text{H}$ ,  $^{125}\text{I}$ ,  $^{35}\text{S}$ ,  $^{14}\text{C}$ , or  $^{32}\text{P}$ ), enzymes (*e.g.*, horse radish peroxidase, alkaline phosphatase and others commonly used in an ELISA), and colorimetric labels such as colloidal gold or colored glass or plastic (*e.g.* polystyrene, polypropylene, latex, *etc.*) beads.

5           The label may be coupled directly or indirectly to the desired component of the assay according to methods well known in the art. As indicated above, a wide variety of labels may be used, with the choice of label depending on sensitivity required, ease of conjugation with the compound, stability requirements, available instrumentation, and disposal provisions..

10           Non-radioactive labels are often attached by indirect means. Generally, a ligand molecule (*e.g.*, biotin) is covalently bound to the molecule. The ligand then binds to an anti-ligand (*e.g.*, streptavidin) molecule which is either inherently detectable or covalently bound to a signal system, such as a detectable enzyme, a fluorescent compound, or a chemiluminescent compound. A number of ligands and anti-ligands can be used. Where a ligand has a natural anti-ligand, for example, biotin, thyroxine, and  
15           cortisol, it can be used in conjunction with the labeled, naturally occurring anti-ligands. Alternatively, any haptenic or antigenic compound can be used in combination with an antibody.

20           The molecules can also be conjugated directly to signal generating compounds, *e.g.*, by conjugation with an enzyme or fluorophore. Enzymes of interest as labels will primarily be hydrolases, particularly phosphatases, esterases and glycosidases, or oxidoreductases, particularly peroxidases. Fluorescent compounds include fluorescein and its derivatives, rhodamine and its derivatives, dansyl, umbelliferone, *etc.* Chemiluminescent compounds include luciferin, and 2,3-dihydrophthalazinediones, *e.g.*,  
25           luminol. For a review of various labeling or signal producing systems which may be used, see, U.S. Patent No. 4,391,904, which is incorporated herein by reference.

30           Means of detecting labels are well known to those of skill in the art. Thus, for example, where the label is a radioactive label, means for detection include a scintillation counter or photographic film as in autoradiography. Where the label is a fluorescent label, it may be detected by exciting the fluorochrome with the appropriate wavelength of light and detecting the resulting fluorescence. The fluorescence may be detected visually, by means of photographic film, by the use of electronic detectors such as charge coupled devices (CCDs) or photomultipliers and the like. Similarly, enzymatic

labels may be detected by providing the appropriate substrates for the enzyme and detecting the resulting reaction product. Finally simple colorimetric labels may be detected simply by observing the color associated with the label. Thus, in various dipstick assays, conjugated gold often appears pink, while various conjugated beads appear the color of the bead.

Some assay formats do not require the use of labeled components. For instance, agglutination assays can be used to detect the presence of the target antibodies. In this case, antigen-coated particles are agglutinated by samples comprising the target antibodies. In this format, none of the components need be labeled and the presence of the target antibody is detected by simple visual inspection.

#### **D) Solid Supports.**

As mentioned above, depending upon the assay, various components, including the nucleic acid probe, antigen, target antibody, or anti-human antibody, may be bound to a solid surface. Many methods for immobilizing biomolecules to a variety of solid surfaces are known in the art. For instance, the solid surface may be a membrane (*e.g.*, nitrocellulose), a microtiter dish (*e.g.*, PVC, polypropylene, or polystyrene), a test tube (glass or plastic), a dipstick (*e.g.* glass, PVC, polypropylene, polystyrene, latex, and the like), a microcentrifuge tube, or a glass or plastic bead. The desired component may be covalently bound or noncovalently attached through nonspecific bonding.

A wide variety of organic and inorganic polymers, both natural and synthetic may be employed as the material for the solid surface. Illustrative polymers include polyethylene, polypropylene, poly(4-methylbutene), polystyrene, polymethacrylate, poly(ethylene terephthalate), rayon, nylon, poly(vinyl butyrate), polyvinylidene difluoride (PVDF), silicones, polyformaldehyde, cellulose, cellulose acetate, nitrocellulose, and the like. Other materials which may be employed, include paper, glasses, ceramics, metals, metalloids, semiconductive materials, cements or the like. In addition, are included substances that form gels, such as proteins (*e.g.*, gelatins), lipopolysaccharides, silicates, agarose and polyacrylamides can be used. Polymers which form several aqueous phases, such as dextrans, polyalkylene glycols or surfactants, such as phospholipids, long chain (12-24 carbon atoms) alkyl ammonium

salts and the like are also suitable. Where the solid surface is porous, various pore sizes may be employed depending upon the nature of the system.

In preparing the surface, a plurality of different materials may be employed, particularly as laminates, to obtain various properties. For example, protein coatings, such as gelatin can be used to avoid non-specific binding, simplify covalent conjugation, enhance signal detection or the like.

If covalent bonding between a compound and the surface is desired, the surface will usually be polyfunctional or be capable of being polyfunctionalized. Functional groups which may be present on the surface and used for linking can include carboxylic acids, aldehydes, amino groups, cyano groups, ethylenic groups, hydroxyl groups, mercapto groups and the like. The manner of linking a wide variety of compounds to various surfaces is well known and is amply illustrated in the literature. See, for example, *Immobilized Enzymes*, Ichiro Chibata, Halsted Press, New York, 1978, and Cuatrecasas, *J. Biol. Chem.* 245 3059 (1970) which are incorporated herein by reference.

In addition to covalent bonding, various methods for noncovalently binding an assay component can be used. Noncovalent binding is typically nonspecific absorption of a compound to the surface. Typically, the surface is blocked with a second compound to prevent nonspecific binding of labeled assay components. Alternatively, the surface is designed such that it nonspecifically binds one component but does not significantly bind another. For example, a surface bearing a lectin such as Concanavalin A will bind a carbohydrate containing compound but not a labeled protein that lacks glycosylation. Various solid surfaces for use in noncovalent attachment of assay components are reviewed in U.S. Patent Nos. 4,447,576 and 4,254,082, which are incorporated herein by reference.

#### **E. Determination of baseline trophoblast-released and/or chorionic villus-released protein levels.**

The abnormal maternal-placental interface is detected by a statistically significant alteration in the concentration of one or more of the trophoblast-released and/or chorionic villus-released proteins of the present invention in a particular assay format as compared to the concentration of the same protein determined in the same assay format for a sample from a mammal having a healthy maternal-placental interface.

Healthy, baseline levels of various trophoblast- or chorionic villus-released proteins may be determined by routine screening of samples isolated from mammals having a healthy maternal-placental interface using any of the assay methods described above followed by standard statistical analysis using methods well known to those of skill in the art.

#### **IV. Screening for Compounds that Mitigate Effects of an Abnormal Placental-Maternal Interface.**

The *in vitro* model provided by the present invention may be used to screen for therapeutic agents that mitigate the development or effects of an abnormal maternal-placental interface. In one embodiment, these method involve culturing hypoxic trophoblasts or chorionic villi, as described above, in the presence of a potential therapeutic agent and measuring changes in the invasiveness of the trophoblasts. An increase of invasiveness would indicate that the therapeutic agent, may aid in restoration of a normal trophoblast phenotype and hence restoration of normal placental function when utilized *in vivo*.

Methods of quantifying trophoblast invasiveness are described by Librach *et al.*, *J. Cell. Biol.*, 113: 437-449 (1991). Briefly, cytotrophoblasts are plated on Matrigel coated polycarbonate filters. After culture for a defined period of time, the samples are fixed, dehydrated and processed for electron microscopy. Cells penetrating the Matrigel surface can then be quantified from the electron microscope images.

Alternatively, one may screen hypoxic trophoblasts or hypoxic chorionic villi for the effect of potential therapeutic agents on the expression level of the proteins whose expression is altered under hypoxic conditions. It is believed that proteins secreted by the trophoblasts and/or chorionic villi are responsible for numerous maternal complications associated with an abnormal maternal-placental interface. Restoration (*e.g.* downregulation) of normal expression of these proteins will mitigate or eliminate diseases of pregnancy caused by these proteins.

One of skill in the art will appreciate that a wide variety of therapeutic agents may be screened for their ability to block, alter, or otherwise prevent the action of released proteins. Such agents include, but are not limited to, agents that inhibit the activity of the secreted proteins such as metalloproteinase inhibitors or serine protease inhibitors, agents (*e.g.* antibodies, lectins, or other ligands) that bind to and inactivate the TSP or tag the TSP protein for destruction by the immune system. Alternatively, the

agent may act on the expression of the TSP from its respective nucleic acid. Such agents may include suppressor genes, antisense molecules, nucleases, and ribozymes.

#### **IV. Screening for Compounds that Adversely Effect the Maternal-Placental Interface.**

Just as there exist agents that mitigate the effects of an abnormal maternal-placental interface, there are other compositions that may adversely effect trophoblast differentiation in a pregnant mammal and thereby induce the formation of an abnormal maternal-placental interface. Clearly it is desirable to identify properties of compounds that may adversely effect development in this manner.

This invention thus provides for methods of screening for agents that might induce the formation of an abnormal maternal-placental interface, or abnormal placental function. In this case, the trophoblasts, or chorionic villi, are cultured under normal conditions and assayed for changes in phenotype as compared to normal and/or hypoxic trophoblasts. Phenotypic changes similar to hypoxic trophoblasts or hypoxic chorionic villi are indicative of possible interference or alteration of the maternal-placental interface by the agent. As described above, the assay may comprise measuring the changes in the levels of expression of one or more proteins expressed by the trophoblasts or chorionic villi. Proteins whose expression is typically altered in hypoxic trophoblasts or hypoxic chorionic villi are preferably assayed, while the proteins listed in Table 1 are most preferably assayed.

#### **VII. Kits for the Detection of Abnormal Placental Function**

The present invention also provides for kits for the diagnosis of women having an abnormal maternal-placental interface and therefore at risk for diseases of pregnancy such as threatened abortion, intrauterine growth retardation, gestational trophoblast diseases including molar pregnancy, choriocarcinoma, placental site tumors, ectopic pregnancy, proteinuria, pregnancy induced hypertension and preeclampsia. The kits preferably include an antibody that specifically binds to one of the trophoblast-released or chorionic villus-released proteins of the present invention or a nucleic acid that specifically hybridizes to another nucleic acid that encodes one or more of the TSPs or chorionic villus-released proteins. The antibody may be free or immobilized on a solid support such as a test tube, a microtiter plate, a dipstick and the like. The kit may also



contain instructional materials teaching the use of the antibody or nucleic acid in an assay for the detection of an abnormal maternal-placental interface.

Additionally, the kit may contain a second antibody that specifically binds the TSP. The second antibody may be labeled, or alternatively, the kit may contain a labeled third antibody that specifically binds the second antibody. The kit may also contain appropriate control series of TSP, buffer solutions, positive and negative controls, washing solutions, dilution buffers and the like for the preparation and analysis of the TSPs in blood or other biological samples..

#### VIII. Method of Modeling Invasive Cells.

The normal human cell type most closely resembling the phenotype of a cancer cell is the placental trophoblast (Manes, *Cancer Res.*, 34: 2044-2052 (1974)), which shares with malignant cells the ability to invade other tissues, to metastasize, and to evade the body's immune response. Only in rare instances, however do trophoblasts become truly malignant as choriocarcinoma. A wide range of cancer cells secrete hormones and proteins characteristic of trophoblasts, and it has been proposed that malignancy is, in part, a pathological recapitulation of normal placental development (Conway, *J. Theoret. Biol.*, 100: 1-24 (1983)).

Thus, the *in vitro* model of the present invention may also be used as a general model for invasive cells types, in particular for invasive cancer cells. Thus the hypoxic trophoblasts of the present invention may also be used to screen for therapeutic agents that inhibit invasiveness and therefore reduce metastases. As described above, the hypoxic trophoblasts may be used to screen changes in invasiveness or, alternatively, for alterations in the expression of proteins that mediate the invasive activity of the cells.

The hypoxic trophoblasts of the present invention may also be used to identify previously unknown proteins found on cancer, in particular invasive cancer, cells. First, proteins whose expression is altered in hypoxic trophoblasts, are identified as described above. Then cancer cells are screened for the presence of the same or closely related proteins. Methods of screening are well known to those of skill in the art. Preferred methods involve screening for immunologic cross reactivity with antibodies raised against the trophoblast proteins. Alternatively, cancer cell mRNA may be screened for sequences that hybridize to nucleic acid probes complementary to nucleic acid sequences or subsequences that encode the trophoblast secreted proteins.

## EXAMPLES

The following examples are offered to illustrate, but not to limit the present invention.

### Example 1

#### Culturing Cytotrophoblasts in an Hypoxic Environment Changes Their Antigenic Phenotype to That Seen in Preeclampsia

Highly purified populations of human cytotrophoblasts are isolated as described by Librach *et al.*, *J. Cell. Biol.*, 113: 437-449 (1991). Briefly, cytotrophoblasts are isolated from first, second and third trimester human placentas according to the methods of Fisher, *et al. J. Cell Biol.*, 109: 891-902 (1989), Fisher *et al. Troph. Res.*, 4: 115-138 (1990); and Kliman *et al. Endocrin.*, 118: 1567-1582 (1986), respectively. Enzyme incubation times differ for different lots of collagenase, hyaluronidase, and trypsin. Yields per gram of placentas are about  $0.5 - 1.0 \times 10^6$  cells. Greater than 95% of the cells are cytotrophoblasts, as determined by fluorescent-activated cell sorting.

Remaining leukocytes are removed using an antibody to CD-45, a protein tyrosine phosphatase found on bone marrow-derived cells (Charbonneau *et al. Proc. Nat. Acad. Sci., USA*, 86: 5252-5256 (1989)), but not on cytotrophoblasts. The antibody (Hle; Becton Dickinson and Col, Mountain View, CA, USA, or IgG affinity purified from the GAP 8.3 hybridoma; American Type Culture Collection, Rockville Maryland, USA) is coupled to magnetic beads (Advanced Magnetix Inc., Cambridge, Massachusetts, USA) and mixed with the cytotrophoblast-enriched Percoll gradient fraction at a density of 25 particles/cell. After incubation for 20 minutes at 4°C with occasional gentle mixing, the CD-45-positive cells are removed by means of a Bio-Mag Separator (advanced Magnetix, Inc.).

Cytotrophoblasts are then cultured on basement membrane substrates in either a normoxic or a hypoxic ( $pO_2 = 14$  mm Hg) environment. Cells are cultured in conditioned medium (DMEM H21 containing 2% Nutridoma) for 48 hr. It is important to note that serum is not added to the culture medium. Thus, complications in the analysis caused by the introduction of this major source of protein are avoided.

Control cultures are placed in a standard tissue culture incubator containing 8% carbon dioxide and air. When cultured under hypoxic conditions, cells

are placed in sealed culture chambers containing 10% CO<sub>2</sub>, 0 to 10% O<sub>2</sub> with the balance being nitrogen.

Differentiation of the normoxic cytotrophoblasts is accompanied by acquisition of an invasive phenotype, as well as by up regulation of the expression of integrins  $\alpha 5/\beta 1$  and  $\alpha 1/\beta 1$ , the 92 kDa type IV collagenase and HLA-G. In contrast, hypoxic cytotrophoblasts have a distinctly different antigenic phenotype. They upregulate  $\alpha 5/\beta 1$  expression, showing that they are capable of carrying out at least of a portion of the normal differentiation program. However, they express very low levels of  $\alpha 1/\beta 1$ , the 92 kDa type IV collagenase and HLA-G. The cells also fail to invade. This is the exact same antigenic phenotype that characterizes cytotrophoblast differentiation in preeclampsia and indicates that these culture conditions can be used to identify unique proteins synthesized by hypoxic cytotrophoblasts that are candidates for the toxic placental factors that are thought to produce the maternal syndrome.

#### Example 2

##### Hypoxic Cytotrophoblasts Up-Regulate their Synthesis of a Discrete Set of Proteins

Cytotrophoblasts, cultured in normoxic and hypoxic conditions, as described in Example 1, are labeled by incorporating in the culture medium 50  $\mu$ Ci/ml [<sup>35</sup>S]cysteine and [<sup>35</sup>S]methionine to facilitate the quantitative analysis of newly synthesized proteins. (125  $\mu$ Ci/ml <sup>14</sup>C-labeled amino acids (high specific activity <sup>14</sup>C-methylated mixture could also be used.)

After 48 h, the medium is dialyzed to remove unincorporated radioactivity. Secreted proteins were precipitated in 80% cold acetone, resuspended in gel loading buffer containing 9 M urea and 2% *n*-octylglucoside and then stored at -70°C prior to electrophoresis.

The secreted protein products are analyzed by 2-dimensional (2-D) SDS-PAGE and the labeled proteins visualized by autoradiography. Samples were run on analytical scale 2-D gels as originally described by Patton *et al. Biotechniques* 8: 518(1990), with precautions suggested by Hunkapillar *et al. Methods in Enzymology*, 91: 227 (1983). Briefly, 10  $\mu$ l of sample containing 400,000 dpm are loaded on 1 mm thick first dimension IEF tube gels containing 2.75% ampholytes (2.1, pH 4-8; pH 3-10), and focused under constant voltage to 18,000 volt-hours. Next, the extruded IEF tube gels are loaded on top of 1 mm thick SDS-PAGE slab gels (pH 8.8) and electrophoresed for 5 h under constant power (20 watts/gel). Following electrophoresis, the gels are fixed in

50% methanol containing 10% acetic acid for 12-18 hr, soaked in fluor and exposed to film for various time periods.

Proteins whose expression is altered in hypoxic trophoblasts are listed in Table 1. In three separate experiments, trophoblasts cultured under hypoxia showed at least a fivefold increase in their production of 5 proteins (designated protein A through E in Table 1) ranging in molecular weight from about 21 to about 62 kDa. Additionally, three proteins were identified (proteins E, F and G) whose expression was downregulated in hypoxic trophoblasts.

### Example 3

#### Further Characterization of Proteins Whose Expression is Altered in Hypoxic Cytotrophoblasts

##### A) Quantitative computer-based analysis of 2D gels.

To determine more precisely the effects of hypoxia, fluorographic exposures of control gels from control and experimental cultures are be visually inspected and computer matched and analyzed. Multiple exposures are scanned with a digital imaging camera interfaced to a SUN3/260 computer. The data are analyzed as previously described (Bersini *et al.*, *Electrophoresis*, 11: 232 (1990)) with the PDQUEST software system, a modification of the software originally developed by Garrels, *Meth. Enzymology*, 100: 411 (1983). This system permits the analysis of 1000-2000 protein spots per gel, depending upon the cell-type and the length of time the gels are exposed to film. These spots may range in abundance from >10,000 ppm (1%) of total labeled protein (*e.g.*, abundant proteins such as actin) to <10 ppm for the most faint proteins, detected only after a one-month fluorographic exposure. An abundance of 10 ppm corresponds to approximately 10,000 copies of the protein per cell.

Protein spots that are highly resolved and induced in relative abundance of hypoxia are selected for sequencing using the mass spectrometric methods described below.

##### B) Preparative-scale 2-D gels.

Preparative gels are run to obtain purified preparations containing several hundred picomoles of individual proteins. The methods are similar to those described above for analytical gels with the following modification. To accommodate the loading

of gels with larger amounts of proteins (500-750  $\mu$ g in 50-150  $\mu$ l), samples are concentrated severalfold using a Centricon 10 centrifugal concentrator. Isoelectric focusing is done in 3.0 mm internal diameter glass tubes. In the first dimension IEF gels, ampholytes are increased to 5.5% and acrylamide is increased to 4%. Extruded IEF gels are soaked for 30 min. in pH 6.8 equilibration buffer. The second dimension SDS slab gels each contain a 2 cm stacking gel (pH 6.8), and the IEF gels are sealed on top of the SDS slab gels with agarose. Following electrophoresis, gels are stained with Coomassie blue, the protein spots of interest excised with a scalpel, and the gel plugs stored frozen at 20°C until electroelution.

#### **C) Isolation, purification and quantitation of proteins from 2-D gel plugs.**

Proteins are isolated from 2-D gel plugs using an electroelution apparatus designed by Hunkapillar (1983), *supra*, or Hoefer Scientific Instruments (San Francisco, California, USA). (Previous studies have demonstrated that more protein can be recovered from polyacrylamide gels by electroelution than by electroblotting onto nitrocellulose or polyvinylidene difluoride membranes.) Electroelution is performed at room temperature and the selected apparatus is fitted with dialysis membrane having the appropriate pore size to insure quantitative recovery of proteins while allowing unwanted impurities to pass. Subsequently, a Konigsberg acetone precipitation (Konigsberg *et al. Meth. Enzymol.*, 91: 254 (1983)) is carried out in order to remove Coomassie blue stain and residual SDS.

#### **D) Proteolytic digestion of proteins and separation of the digest components.**

The proteins are enzymatically digested with high purity trypsin (enzyme/substrate ratio = 1:20) in the presence of 4M urea at 37°C. The digestion is carried out for 16 hr to insure complete digestion of the proteins. Separation of the resulting digestion components is achieved on a reserved phase C<sub>18</sub>, microbore (1.0 mm diameter x 10 cm) HPLC column which, prior to sample injection, is equilibrated with 0.1% trifluoroacetic acid (TFA) in water (Solvent A). Isocratic elution with Solvent A for 10 minutes is followed by a linear gradient (0.5% per min.) to a final mobile phase composition of 70% acetonitrile/30% water/0.1% TFA at a flow rate of 50  $\mu$ l/min. Peptide elution is monitored at 215 nm and fractions are collected either manually or with an automated fraction collector.

**E) Mass spectrometric sequencing of peptides obtained from proteolytic digestion:  
Molecular weight mapping of separated proteolytic digest components.**

Matrix assisted laser desorption ionization (MALDI) is used (Hall *et al.*, *Proc. Nat. Acad. Sci. USA*, 90: 1927 (1993)). This highly sensitive technique allows  
5 determination of the peptide molecular mass for each HPLC fraction from as little as 1/50 of the entire sample.

Once the molecular weight of each tryptic peptide is determined, high energy collision induced dissociation (CID) mass spectra is determined from the remainder of each HPLC fraction. When some fractions contain multiple components an  
10 advantage of tandem mass spectrometric over Edman sequencing is that each component from such a mixture is selected and sequenced in turn. A high performance Kratos Concept IHH four sector tandem mass spectrometer (Kratos Instruments, Ramsey, New Jersey, USA) equipped with a cesium ion source, continuous flow sample introduction and a scanning, charge-coupled device multichannel array detector is used to obtain the  
15 CID mass spectra (Burlingame *et al. Analyt. Chem.*, 66: 634R-683R (1994)).

The first mass spectrometer is used to select the  $^{12}\text{C}$  isobar of the protonated molecular ion of the peptide of interest. This species is then be accelerated into a collision cell, floating at 4keV, where it collides with helium atoms. The resulting collisions impart sufficient vibrational energy into the peptide to induce fragmentation.  
20 These fragments then pass into the second mass spectrometer where they are mass analyzed and their relative abundances recorded. Complete high energy CID mass spectra are recorded every 11 sec. as sample begins to flow into the ion source from a liquid inlet system containing the peptides of interest. This recently developed unique technology optimizes sensitivity and minimizes low level sample losses such that peptide  
25 sequence analysis can be carried out at the subpicomole level. This is achieved using a multi-channel array detection system with a charge-coupled device readout of the spectrum in real time (Burlingame *et al. Analyt. Chem.*, 66: 634R-683R (1994)). The sequence of peptides are deduced with the aid of interactive computer algorithms developed by Hines *et al.*, *J. Am. Soc. Mass. Spectrom.*, 3: 326 (1992).

**F) Computer search of Dayhoff and PIR protein data bases for related sequences of known proteins.**

Peptide sequences deduced from the interpretation of the CID mass spectra are used to search the Dayhoff and PIR protein data bases in an attempt to relate them to known individual proteins or families of proteins.

**F) Confirmation of sequenced proteins induced by hypoxia at the nucleic acid level.**

The sequences of the induced proteins may be confirmed by redundant sequencing either by tandem mass spectrometry or, alternatively, the nucleic acid sequences encoding the proteins are cloned and sequenced using standard techniques known to those of skill in the art.

**Example 4**

**Isolation and Sequencing of Proteins Whose Expression is Altered in Hypoxic Chorionic Villi**

**A) Hypoxia alters chorionic villus release of a discrete number of unlabeled proteins in culture.**

Having used two-dimensional electrophoresis (PAGE), as described above, to measure protein synthesis/secretion changes in hypoxic cytotrophoblasts, a similar experiment was attempted with an alternate model system,; explanted chorionic villi. Anchoring chorionic villi, dissected from first trimester placenta were transferred to a mixture of F12 HAM/DMEM (1:1/v:v) culture medium (H/D medium) containing 10% fetal calf serum (FCS). Initially, FCS was included because of the expectation that it might promote the cells' attachment to the substrate. Six villi/well were plated in Matrigel-coated 35-mm culture dishes. One dish (4-6 wells) was placed in an hypoxic atmosphere (2% O<sub>2</sub>) and the other dish was placed in a standard tissue culture incubater (20% O<sub>2</sub>). After 24 h, the medium was aspirated and replaced with H/D medium that contained 2% Nutridoma. It was expected that the proteins in Nutridoma would be easily distinguished from those released by the villi in subsequent two-dimensional electrophoresis.

After 72 h, culture medium was collected and centrifuged through a Centricon concentrator with a 10 kDa cutoff. The samples (700 µg protein in 50-150 µL) were subjected to PAGE as described above. After electrophoresis, the gels were

stained with colloidal Coomassie Blue Fast Stain (ZOION), then destained by incubation in methanol/acetic acid/H<sub>2</sub>O (45/10/45:v/v/v).

This experiment was repeated four times using villi from different placentas. The results of each were essentially the same. The two-dimensional electrophoresis maps of culture medium samples from cultures maintained under experimental hypoxia and control conditions were nearly identical; the abundance of only a few proteins changed in response to lowering O<sub>2</sub> tension. The results were similar to those shown in Figures 1 and 2 (2D gels for chorionic villi grown in F12 HAM/DMEM, *see* section C, below).

**B) Sequencing of proteins whose expression/secretion is altered in hypoxic chorionic villi.**

Mass spectrometry (MS) sequencing was used to determine the identity of proteins whose abundance changes in hypoxia. This approach was initially applied to one protein spot whose release into culture media was dramatically increased when chorionic villi were cultured under hypoxic conditions. This spot was excised from six gels and subjected to a 5-step in-gel trypsin digestion as follows:

- (1) The gel plugs were macerated to minimize the size of gel particle and to facilitate enzyme access to the trapped protein.
- (2) The gel particles were destained by three washes (15 min each) with 25 mM ammonium bicarbonate/50% acetonitrile.
- (3) Destained particles were dried for 30 min by vacuum centrifugation (in a Speed-Vac).
- (4) The trapped protein was digested by rehydrating the gel particles in 25 mM ammonium bicarbonate buffer containing 0.1 µg/µL high grade trypsin.
- (5) Peptides were recovered by three extractions of the digestion mixture with 50% acetonitrile/5% TFA.

Control digestions of gel plugs without protein were performed so that trypsin autoproteolysis products could be identified and disregarded in the MS spectra. A portion (1/25th) of the digest was co-crystallized in a matrix of α-cyano-4-hydroxycinnamic acid and analyzed using a VG ToFSpec SE Matrix Assisted Laser Desorption Ionization (MALDI) mass spectrometer equipped with a nitrogen laser and



operated in reflectron mode. Digests were analyzed without first being separated by HPLC, thereby saving time and conserving sample. All MALDI spectra were externally calibrated using a standard peptide mixture.

The resulting mass spectra contained two types of information. First a peptide -mass fingerprint was obtained by measuring the monoisotopic molecular masses ( $\pm 0.5$  Da) of several peptides in the digest. Then peptide sequence information was obtained in the form of a peptide fragment ion tag by acquiring MALDI post-source decay spectra following ion-gating of individual peptides. a peptide fragment ion tag could contain a variety of sequence-ion types produced by several different cleavage processes (a, b, y, a-NH<sub>3</sub>, b-NH<sub>3</sub>, y-NH<sub>3</sub>, b-H<sub>2</sub>O, and ammonium ions).

Genomic databases (*e.g.*, GenBank) were searched for known proteins whose peptide-mass fingerprints and peptide sequences matched those in the MALDI spectra of the peptides recovered from the two-dimensional electrophoresis spot.

The results showed the protein was apolipoprotein a-1 (apo a-1). Surprisingly, the protein match was bovine, not human. Evidently the sampled protein came from the medium in which the villi were incubated during the first 24 hours of the 72 hour culture period (H/D with 10% FCS). Nevertheless it was hypothesized that release of the human protein might be similarly regulated.

To determine if this was the case, a mouse monoclonal antibody against human apo a-I was used to analyze, by Western blotting, culture medium samples from chorionic villi maintained under control and experimental hypoxia conditions. In control samples, a band corresponding to the molecular weight of apo a-1 (28 kDa) was barely visible. In contrast, the abundance of this protein was greatly increased in culture medium samples from villi maintained in 2% O<sub>2</sub>. a 17 kDa band, which it is believed corresponds to a fragment produced by cleavage in a proteinase-sensitive region of the molecule was also detected. Higher molecular weight diffuse bands were due to a low level of antibody reactivity with the protein components of Nutridoma. These experiments show that human apo a-I is more abundant in culture media when chorionic villi are cultured under hypoxic conditions.

Using the above-described sequencing methods, other proteins whose expression/secretion levels vary in hypoxic chorionic villi were identified (*see* Table 2). These include, for example, placental lactogen, chorionic gonadotropin, and fibrinogen.

Table 2. Protein expression/secretion in hypoxic chorionic villi.

Spot Number <sup>1</sup>	Change in Hypoxia <sup>2</sup>	Protein Name <sup>3</sup>	Amino acid sequence <sup>4</sup>	Sequence ID NO.
1	down			
2	down	Placental lactogen	LFDHAMLQAHR ISLLIESWLEPVR	1 2
3	down	Placental lactogen	NYGLLYCFR LFDHAMLQAHR ISLLIESWLEPVR AHQLAIDTYQEFETYIPK	3 4 5 6
4	up			
5	up	Apolipoprotein a-1	WHEEVEIYR	7
6	down			
7	up	Chorionic gonadotropin	VLQGVLPALPQVVCNYR	8
8	down			
9	unchanged	78 kDa glucose-regulated protein	FELTAIPPAPR NSLESYAFNMK DNHLLGTFDLTGIPPAPR	9 10 11
10	up	Fibrinogen	LYIDETVNDNIPLNLR IRPFFPQQ	12 13
11	down	Serum albumin	RHPEYAVSVLLR LGEYGFQNALIVR DAFLGSFLYEYSR KVPQVSTPTLVEVSR RPCFSALTPDETYVPK	14 15 16 17 18
12	down	Serum albumin		
13	down	Serum albumin		
14	down			
15	down			
17	up			
19	unchanged		FELTAIPPAPR DNHLLGTFDLTGIPPAPR	19 20

21 up  
22 down

1. See, *e.g.*, Figure 1 and Figure 2 for identification of spots. A protein of spot n (where n is the spot number) will appear in the same position relative to the other spots as the spot n in Figure 1 and/or Figure 2.
2. Change in abundance of protein released by hypoxic chorionic villi cultured under 2% O<sub>2</sub>.
3. Nominal protein identity as determined by a search of GenBank. It is recognized that while the sequenced fragments share identity with the identified protein, the protein abnormally expressed under hypoxic conditions can also be a fragment, a mutant, a post-translationally modified variant, a species variant, or another variant of the identified protein.
4. Amino acid sequence(s) of tryptic fragments of protein extracted from the spot.
5. The same as spot 11 by peptide mass fingerprint.
6. Identified in hypoxic chorionic villi cultured in FCS.

### **C) Immunolocalization of apo a-I.**

Immunohistochemistry was used to confirm the predicted changes in placental protein level changes in preeclampsia. Accordingly, the antihuman apo a-I antibody was used to stain tissue sections of the placenta and placental bed of preeclamptic (n=5) and control (n=4) patients. Immunolocalization of apo a-I was performed essentially as described by Zhou *et al.* (1993). To identify trophoblasts, the sections were double stained with an antibody that recognized cytokeratin (CK).

In normal pregnancy, the apo a-I antibody reacted with various elements of the floating chorionic villi, including cytotrophoblasts and the syncytial brush border. This observation is consistent with experimental evidence showing that the placenta expresses an apo a-I binding protein (Ehnholm *et al.* (1991)).

Elements of the stromal core were also stained, although they were weak and diffuse. In preeclampsia, placental staining for apo a-I was greatly enhanced in all locations. Within chorionic villi, elements of the stromal core were clearly defined by antibody reactivity. Blood vessels were particularly well outlined. Within the placental bed, cytotrophoblast staining was easily seen.

These experiments show that hypoxic cytotrophoblasts and chorionic villi provide a useful model system for the abnormal maternal/placenta interface. Proteins whose regulation varies with hypoxia and which appear to be good markers for the identification (diagnosis) of the abnormal maternal/placental interface and attendant pathology (*e.g.*, preeclampsia) were identified.

**D) Alteration of culture media.**

In addition, the results of these experiments suggest that placental cells take up proteins from the medium and these proteins can be later released. Although the described sequencing methods can easily determine whether 2D gel spots are bovine or human proteins, culturing the cells in defined media avoids this complication. Therefore, cell attachment and protein release by chorionic villi cultured in the following treatments:

- (1) H/D containing 10% FCS for the first 24 hours, then switched to H/D containing 2% Nutridoma for the last 48 hours (as described above);
- (2) H/D with 2% Nutridoma for 72 hours; and
- (3) H/D for 72 hours.

These experiments were repeated twice with the same results. Attachment and appearance of the villi did not differ in any of the media. In addition, all three conditions gave essentially the same 2D maps except that the spot which contained bovine apo a-I was missing in samples prepared in medium 2 and Nutridoma protein additives were missing in culture media samples prepared in medium 3.

Therefore, in a preferred embodiment, chorionic villi are cultured in F12 HAM/DMEM (1:1:v:v) -- medium 3. An illustration of two-dimensional electrophoresis gels produced from chorionic villus cultures in medium lacking FCS under normoxic and hypoxic conditions respectively are provided in Figures 1 and 2, respectively.

In summary, the experiments described above indicate that hypoxia changes the repertoire of substances the placenta synthesizes and/or releases. These factors include molecules whose expression is known to be regulated by O<sub>2</sub> tension in other cells (interleukins and growth factors), known proteins whose abundance was not previously known to change in preeclampsia (*e.g.*, apo a-I), and proteins that have yet to be sequenced.

It is understood that the examples and embodiments described herein are for illustrative purposes only and that various modifications or changes in light thereof will be suggested to persons skilled in the art and are to be included within the spirit and purview of this application and scope of the appended claims. All publications, patents, and patent applications cited herein are hereby incorporated by reference.

**WHAT IS CLAIMED IS:**

1                   1. A protein released by a mammalian fetal trophoblast cell or a  
2 chorionic villus wherein the level of release is substantially changed when the  
3 cell or villus is grown under hypoxic conditions characterized by a partial  
4 pressure of oxygen ( $pO_2$ ) of 14 mm of mercury (mm Hg), wherein said protein  
5 is selected from the group of proteins consisting of:

6                   (a) Protein A having a molecular weight of about 21 kDa and a  
7 pI of 6.0 wherein the release of said protein, under hypoxic conditions is  
8 increased;

9                   (b) Protein B having a molecular weight of about 22 kDa and a pI  
10 of 7.0 wherein the release of said protein, under hypoxic conditions is  
11 increased;

12                   (c) Protein C having a molecular weight of about 23 kDa and a pI  
13 of 7.5 wherein the release of said protein, under hypoxic conditions, is  
14 increased;

15                   (d) Protein D having a molecular weight of about 55 kDa and a  
16 pI of 8.5 wherein the release of said protein, under hypoxic conditions, is  
17 increased;

18                   (e) Protein E having a molecular weight of about 62 kDa and a pI  
19 of 5.5 wherein the release of said protein, under hypoxic conditions, is  
20 increased;

21                   (f) Protein F having a molecular weight of about 40 kDa and a pI  
22 of 4.5 wherein the release of said protein, under hypoxic conditions, is  
23 decreased;

24                   (g) Protein G having a molecular weight of about 67 kDa and a  
25 pI of 6.5 wherein the release of said protein, under hypoxic conditions, is  
26 decreased;

27                   (h) Protein H having a molecular weight of about 75 kDa and a  
28 pI of 9.0 wherein the release of said protein, under hypoxic conditions, is  
29 decreased;

30                   (i) A protein of spot number 2 comprising an amino acid  
31 sequence selected from the group consisting of sequence 1, and sequence 2 as  
32 shown in Table 2;

(j) A protein of spot number 3 comprising an amino acid sequence selected from the group consisting of sequence 3, sequence 4, sequence 5, and sequence 6 as shown in Table 2;

(k) A protein of spot number 5 comprising amino acid sequence number 7 as shown in Table 2;

(l) A protein of spot number 7 comprising amino acid sequence number 8 as shown in Table 2;

(m) A protein of spot number 10 comprising an amino acid sequence selected from the group consisting of sequence sequence 12, and sequence 13 as shown in Table 2;

(n) A protein of spot number 11 comprising an amino acid sequence selected from the group consisting of sequence 14, sequence 15, sequence 16, sequence 17, and sequence 18 as shown in Table 2; and

(o) A protein of spot number 20 comprising an amino acid sequence selected from the group consisting of sequence 21, and sequence 22 as shown in Table 2; and

(p) A human apolipoprotein A-1.

2. A protein of claim 1, wherein the protein is selected from the group consisting of:

(a) Protein A having a molecular weight of about 21 kDa and a pI of 6.0 wherein the release of said protein, under hypoxic conditions, is increased;

(b) Protein B having a molecular weight of about 22 kDa and a pI of 7.0 wherein the release of said protein, under hypoxic conditions, is increased;

(c) Protein C having a molecular weight of about 23 kDa and a pI of 7.5 wherein the release of said protein, under hypoxic conditions, is increased;

(d) Protein D having a molecular weight of about 55 kDa and a pI of 8.5 wherein the release of said protein, under hypoxic conditions, is increased; and,

(e) Protein E having a molecular weight of about 62 kDa and a pI of 5.5 wherein the release of said protein, under hypoxic conditions, is increased.

3. A protein of claim 1, wherein the protein is selected from the group consisting of:

(f) Protein F having a molecular weight of about 40 kDa and a pI of 4.5 wherein the release of said protein, under hypoxic conditions, is decreased;

(g) Protein G having a molecular weight of about 67 kDa and a pI of 6.5 wherein the release of said protein, under hypoxic conditions, is decreased; and

(h) Protein H having a molecular weight of about 75 kDa and a pI of 9.0 wherein the release of said protein, under hypoxic conditions, is decreased.

4. A protein of claim 1, wherein the protein is selected from the group consisting of:

(i) A protein of spot number 2 comprising an amino acid sequence selected from the group consisting of sequence 1, and sequence 2 as shown in Table 2 and wherein the release of said protein, under hypoxic conditions, is decreased;

(j) A protein of spot number 3 comprising an amino acid sequences selected from the group consisting of sequence 3, sequence 4, sequence 5, and sequence 6 as shown in Table 2 and wherein the release of said protein, under hypoxic conditions, is decreased;

(k) A protein of spot number 5 comprising amino acid sequence number 7 as shown in Table 2 and wherein the release of said protein, under hypoxic conditions, is increased;

(l) A protein of spot number 7 comprising amino acid sequence number 8 as shown in Table 2 and wherein the release of said protein, under hypoxic conditions, is increased;

(m) A protein of spot number 10 comprising an amino acid sequence selected from the group consisting of sequence 12, and sequence 13 as shown in Table 2 and wherein the release of said protein, under hypoxic conditions, is increased;

(n) A protein of spot number 11 comprising an amino acid sequence selected from the group consisting of sequence 14, sequence 15, sequence 16, sequence 17, and sequence 18 as shown in Table 2 and wherein the release of said protein, under hypoxic conditions, is decreased; and

(o) A protein of spot number 20 comprising an amino acid sequence selected from the group consisting of sequence 21, and sequence 22 as shown in Table 2 and wherein the release of said protein, under hypoxic conditions, is increased; and

(p) A human apolipoprotein A-1 wherein the release of said protein, under hypoxic conditions, is increased.

5. A method of culturing human fetal trophoblast cells or chorionic villi under hypoxic conditions, said method comprising the step of culturing the trophoblast cells or chorionic villi under an atmosphere comprising less than about 20% oxygen.

6. A method of claim 5, wherein the method further comprises measuring the release of a protein selected from the group consisting of:

(a) Protein A having a molecular weight of about 21 kDa and a pI of 6.0 wherein the release of said protein, under hypoxic conditions, is increased;

(b) Protein B having a molecular weight of about 22 kDa and a pI of 7.0 wherein the release of said protein, under hypoxic conditions, is increased;

(c) Protein C having a molecular weight of about 23 kDa and a pI of 7.5 wherein the release of said protein, under hypoxic conditions, is increased;



(d) Protein D having a molecular weight of about 55 kDa and a pI of 8.5 wherein the release of said protein, under hypoxic conditions, is increased;

(e) Protein E having a molecular weight of about 62 kDa and a pI of 5.5 wherein the release of said protein, under hypoxic conditions, is increased;

(f) Protein F having a molecular weight of about 40 kDa and a pI of 4.5 wherein the release said protein, under hypoxic conditions, is decreased;

(g) Protein G having a molecular weight of about 67 kDa and a pI of 6.5 wherein the release of said protein, under hypoxic conditions, is decreased; and

(h) Protein H having a molecular weight of about 75 kDa and a pI of 9.0 wherein the release of said protein, under hypoxic conditions, is decreased;

(i) A protein of spot number 2 comprising an amino acid sequence selected from the group consisting of sequence 1, and sequence 2 as shown in Table 2 and wherein the release of said protein, under hypoxic conditions, is decreased;

(j) A protein of spot number 3 comprising an amino acid sequences selected from the group consisting of sequence 3, sequence 4, sequence 5, and sequence 6 as shown in Table 2 and wherein the release of said protein, under hypoxic conditions, is decreased;

(k) A protein of spot number 5 comprising amino acid sequence number 7 as shown in Table 2 and wherein the release of said protein, under hypoxic conditions, is increased;

(l) A protein of spot number 7 comprising amino acid sequence number 8 as shown in Table 2 and wherein the release of said protein, under hypoxic conditions, is increased;

(m) A protein of spot number 10 comprising an amino acid sequence selected from the group consisting of sequence 12, and sequence 13 as shown in Table 2 and wherein the release of said protein, under hypoxic conditions, is increased;

(n) A protein of spot number 11 comprising an amino acid sequence selected from the group consisting of sequence 14, sequence 15, sequence 16, sequence 17, and sequence 18 as shown in Table 2 and wherein the release of said protein, under hypoxic conditions, is decreased; and

(o) A protein of spot number 20 comprising an amino acid sequence selected from the group consisting of sequence 21, and sequence 22 as shown in Table 2 and wherein the release of said protein, under hypoxic conditions, is increased; and

(p) A human apolipoprotein A-1 wherein the release of said protein, under hypoxic conditions, is increased.

7. A method of detecting hypoxic cytotrophoblast cells or hypoxic chorionic villi, said method comprising measuring the release of a protein selected from the group consisting of:

(a) Protein A having a molecular weight of about 21 kDa and a pI of 6.0 wherein the release of said protein, under hypoxic conditions, is increased;

(b) Protein B having a molecular weight of about 22 kDa and a pI of 7.0 wherein the release of said protein, under hypoxic conditions, is increased;

(c) Protein C having a molecular weight of about 23 kDa and a pI of 7.5 wherein the release of said protein, under hypoxic conditions, is increased;

(d) Protein D having a molecular weight of about 55 kDa and a pI of 8.5 wherein the release of said protein, under hypoxic conditions, is increased;

(e) Protein E having a molecular weight of about 62 kDa and a pI of 5.5 wherein the release of said protein, under hypoxic conditions, is increased;

(f) Protein F having a molecular weight of about 40 kDa and a pI of 4.5 wherein the release of said protein, under hypoxic conditions, is decreased;

(g) Protein G having a molecular weight of about 67 kDa and a pI of 6.5 wherein the release of said protein, under hypoxic conditions, is decreased; and

(h) Protein H having a molecular weight of about 75 kDa and a pI of 9.0 wherein the release of said protein, under hypoxic conditions, is decreased;

(i) A protein of spot number 2 comprising an amino acid sequence selected from the group consisting of sequence 1, and sequence 2 as shown in Table 2 and wherein the release of said protein, under hypoxic conditions, is decreased;

(j) A protein of spot number 3 comprising an amino acid sequences selected from the group consisting of sequence 3, sequence 4, sequence 5, and sequence 6 as shown in Table 2 and wherein the release of said protein, under hypoxic conditions, is decreased;

(k) A protein of spot number 5 comprising amino acid sequence number 7 as shown in Table 2 and wherein the release of said protein, under hypoxic conditions, is increased;

(l) A protein of spot number 7 comprising amino acid sequence number 8 as shown in Table 2 and wherein the release of said protein, under hypoxic conditions, is increased;

(m) A protein of spot number 10 comprising an amino acid sequence selected from the group consisting of sequence 12, and sequence 13 as shown in Table 2 and wherein the release of said protein, under hypoxic conditions, is increased;

(n) A protein of spot number 11 comprising an amino acid sequence selected from the group consisting of sequence 14, sequence 15, sequence 16, sequence 17, and sequence 18 as shown in Table 2 and wherein the release of said protein, under hypoxic conditions, is decreased; and

(o) A protein of spot number 20 comprising an amino acid sequence selected from the group consisting of sequence 21, and sequence 22 as shown in Table 2 and wherein the release of said protein, under hypoxic conditions, is increased; and

(p) A human apolipoprotein A-1 wherein the release of said protein, under hypoxic conditions, is increased;  
wherein the release of the protein is increased or decreased relative to identical cells grown under identical culture conditions but under normal oxygen conditions.

8. A method of claim 7, wherein the measurement is by direct determination of the protein.

9. A method of claim 7, wherein the determination comprises the step of binding an antibody to the protein and determining the quantity of bound antibody present in a sample relative to the quantity of antibody bound to protein obtained from normoxic trophoblasts or normoxic chorionic villi.

10. A method of claim 7, wherein the determination comprises detecting mRNA encoding any of the proteins and determining if the level of mRNA has changed relative to similarly treated normoxic cells.

11. A method for detecting an abnormal placental function by analysing a biological sample from a pregnant mammal for abnormal release of a protein selected from the group consisting of:

(a) Protein A having a molecular weight of about 21 kDa and a pI of 6.0 wherein the release of said protein, under hypoxic conditions, is increased;

(b) Protein B having a molecular weight of about 22 kDa and a pI of 7.0 wherein the release of said protein, under hypoxic conditions, is increased;

(c) Protein C having a molecular weight of about 23 kDa and a pI of 7.5 wherein the release of said protein, under hypoxic conditions, is increased;

(d) Protein D having a molecular weight of about 55 kDa and a pI of 8.5 wherein the release of said protein, under hypoxic conditions, is increased;

1 (e) Protein E having a molecular weight of about 62 kDa and a pI  
2 of 5.5 wherein the release of said protein, under hypoxic conditions, is  
3 increased;

4 (f) Protein F having a molecular weight of about 40 kDa and a pI  
5 of 4.5 wherein the release of said protein, under hypoxic conditions, is  
6 decreased;

7 (g) Protein G having a molecular weight of about 67 kDa and a  
8 pI of 6.5 wherein the release of said protein, under hypoxic conditions, is  
9 decreased; and,

10 (h) Protein H having a molecular weight of about 75 kDa and a  
11 pI of 9.0 wherein the release of said protein, under hypoxic conditions, is  
12 decreased;

13 (i) A protein of spot number 2 comprising an amino acid  
14 sequence selected from the group consisting of sequence 1, and sequence 2 as  
15 shown in Table 2 and wherein the release of said protein, under hypoxic  
16 conditions, is decreased;

17 (j) A protein of spot number 3 comprising an amino acid  
18 sequences selected from the group consisting of sequence 3, sequence 4,  
19 sequence 5, and sequence 6 as shown in Table 2 and wherein the release of said  
20 protein, under hypoxic conditions, is decreased;

21 (k) A protein of spot number 5 comprising amino acid sequence  
22 number 7 as shown in Table 2 and wherein the release of said protein, under  
23 hypoxic conditions, is increased;

24 (l) A protein of spot number 7 comprising amino acid sequence  
25 number 8 as shown in Table 2 and wherein the release of said protein, under  
26 hypoxic conditions, is increased;

27 (m) A protein of spot number 10 comprising an amino acid  
28 sequence selected from the group consisting of sequence 12, and sequence 13  
29 as shown in Table 2 and wherein the release of said protein, under hypoxic  
30 conditions, is increased;

31 (n) A protein of spot number 11 comprising an amino acid  
32 sequence selected from the group consisting of sequence 14, sequence 15,

1 sequence 16, sequence 17, and sequence 18 as shown in Table 2 and wherein  
2 the release of said protein, under hypoxic conditions, is decreased; and

3 (o) A protein of spot number 20 comprising an amino acid  
4 sequence selected from the group consisting of sequence 21, and sequence 22 as  
5 shown in Table 2 and wherein the release of said protein, under hypoxic  
6 conditions, is increased; and

7 (p) A human apolipoprotein A-1 wherein the release of said  
8 protein, under hypoxic conditions, is increased..

1 12. A method of claim 11, wherein said abnormal placental  
2 function is a symptom of a disease of pregnancy selected from the group  
3 consisting of threatened abortion, intrauterine growth retardation, gestational  
4 trophoblast diseases including molar pregnancy, choriocarcinoma, placental site  
5 tumors, ectopic pregnancy, proteinuria, pregnancy induced hypertension and  
6 preeclampsia.

1 13. A method of claim 12, wherein said disease of pregnancy is  
2 preeclampsia.

1 14. A method of screening for agents that mitigate the effects of  
2 an abnormal maternal-placental interface, said method comprising:

3 (i) culturing cytotrophoblasts under hypoxic conditions in the  
4 presence of said agent; and

5 (ii) assaying for changes in the phenotype of said hypoxic  
6 trophoblasts relative to hypoxic trophoblasts cultures without the presence of  
7 said agent.

1 15. The method of claim 14, wherein said assaying comprises  
2 measuring the invasiveness of said trophoblasts.

1 16. The method of claim 14, wherein said assaying comprises  
2 measuring the changes in the levels of release of proteins expressed by said  
3 trophoblasts.

1           17. The method of modeling, *in vitro*, an abnormal maternal-  
2 placental interface, said method comprising culturing trophoblast cells or  
3 chorionic villi in a hypoxic environment.

1           18. The method of claim 17, wherein said hypoxic environment  
2 comprises an atmosphere comprising less than about 20% oxygen.

1           19. A method for identifying proteins that are indicative of  
2 metastasis said method comprising:

- 3           (i) raising cytotrophoblasts under hypoxic conditions; and  
4           (ii) detecting proteins that demonstrate an altered release level as  
5 a result of said hypoxic conditions; and,  
6           (iii) determining if said proteins are present in metastatic cells.

7           20. A method of claim 19, wherein the determining is done by  
8 immunoassay using antibodies specific for at least one of the proteins of step ii.

9           21. A method for identifying proteins that are indicative of an  
10 abnormal maternal placental interface said method comprising:

- 11           (i) culturing cytotrophoblasts under hypoxic conditions; and  
12           (ii) detecting proteins that demonstrate an altered release level as  
13 a result of the hypoxic conditions.

1           22. A method of claim 21, wherein said abnormal maternal  
2 placental interface is indicative of preeclampsia.

1/2

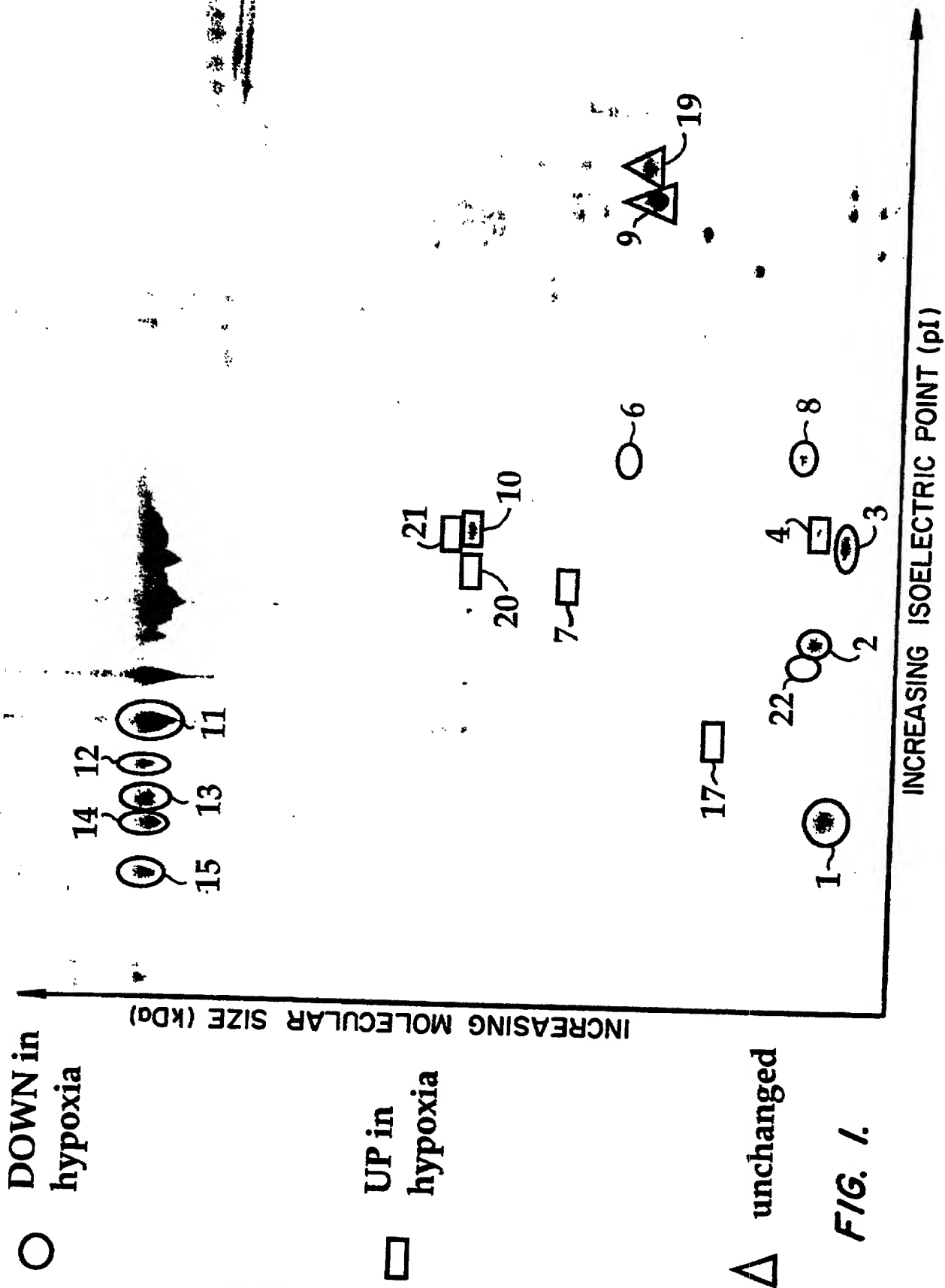


FIG. 1.



2/2

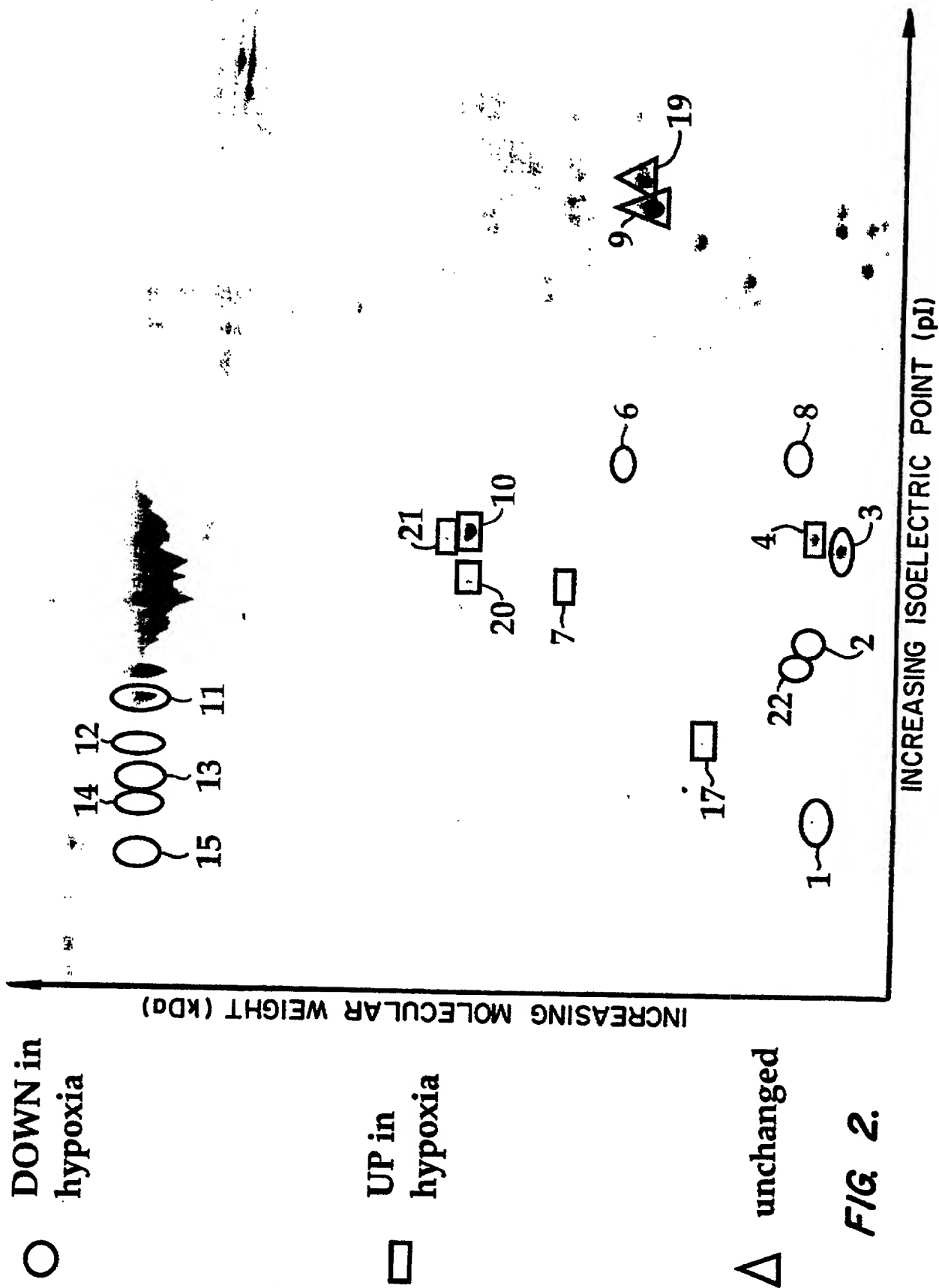


FIG. 2.

02307E-060010US

As a below-named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name,

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled:

ALTERED PROTEIN EXPRESSION IN HYPOXIC TROPHOBLASTS

the specification of which (check only one item below):

☐ is attached hereto.

☐ was filed as United States application

Serial No. \_\_\_\_\_

on \_\_\_\_\_,

and was amended

on \_\_\_\_\_ (if applicable).

☒ was filed as PCT international application

Number \_\_\_\_\_ PCT/US96/05441

on \_\_\_\_\_ April 18, 1996

and was amended under PCT Article 19

on \_\_\_\_\_ (if applicable).

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information which is material to the examination of this application in accordance with Title 37, Code of Federal Regulations, §1.56(a).

I hereby claim foreign priority benefits under Title 35, United States Code, §119 of any foreign application(s) for patent or inventor's certificate or of any PCT international application(s) designating at least one country other than the United States of America listed below and have also identified below any foreign application(s) for patent or inventor's certificate or any PCT international application(s) designating at least one country other than the United States of America filed by me on the same subject matter having a filing date before that of the application(s) of which priority is claimed:

## PRIOR FOREIGN/PCT APPLICATION(S) AND ANY PRIORITY CLAIMS UNDER 35 U.S.C. 119:

COUNTRY (if PCT, indicate "PCT")	APPLICATION NUMBER	DATE OF FILING (day, month, year)	PRIORITY CLAIMED UNDER 35 USC 119	
US	08/423,409	April 18, 1995	<input checked="" type="checkbox"/> YES	<input type="checkbox"/> NO
			<input type="checkbox"/> YES	<input type="checkbox"/> NO
			<input type="checkbox"/> YES	<input type="checkbox"/> NO
			<input type="checkbox"/> YES	<input type="checkbox"/> NO
			<input type="checkbox"/> YES	<input type="checkbox"/> NO

TOWNSEND SF

ATTORNEY'S DOCKET NUMBER  
02307E-060010US

I hereby claim the benefit under Title 35, United States Code, §120 of any United States application(s) or PCT international application(s) designating the United States of America that is/are listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in that/those prior application(s) in the manner provided by the first paragraph of Title 35, United States Code, §112, I acknowledge the duty to disclose material information as defined in Title 37, Code of Federal Regulations, §1.56(a) which occurred between the filing date of the prior application(s) and the national or PCT international filing date of this application:

PRIOR U.S. APPLICATIONS OR PCT INTERNATIONAL APPLICATIONS DESIGNATING THE U.S. FOR BENEFIT UNDER 35 U.S.C. 120:

U.S. APPLICATIONS			STATUS (Check one)		
U.S. APPLICATION NUMBER	U.S. FILING DATE	PATENTED	PENDING	ABANDONED	
PCT APPLICATIONS DESIGNATING THE U.S.					
PCT APPLICATION NO.	PCT FILING DATE	U.S. SERIAL NUMBERS ASSIGNED (if any)			

**POWER OF ATTORNEY:** As a named inventor, I hereby appoint the following attorney(s) and/or agent(s) to prosecute this application and transact all business in the Patent and Trademark Office connected therewith. *(List name and registration number)*

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I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

SIGNATURE OF INVENTOR 203

DATE

PRIOR U.S. APPLICATIONS OR PCT INTERNATIONAL APPLICATIONS DESIGNATING THE U.S. FOR BENEFIT UNDER 35 U.S.C. 120:

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Tom Hunter, Reg. No. 38,498

**Direct Telephone Calls to:**  
*(name and telephone number)*  
Tom Hunter  
415-576-0200

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

**SIGNATURE OF INVENTOR 203**

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01/05/98 MON 11:01 FAX 415 578 0300

TTC

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	POST OFFICE ADDRESS	POST OFFICE ADDRESS	CITY	STATE & ZIP CODE/COUNTRY
<p>I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.</p>				
SIGNATURE OF INVENTOR 204		SIGNATURE OF INVENTOR 205		SIGNATURE OF INVENTOR 206
DATE 1/5/98		DATE		DATE

204	FULL NAME OF INVENTOR	FAMILY NAME CLAUSER	FIRST GIVEN NAME Karl	SECOND GIVEN NAME R.
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206	FULL NAME OF INVENTOR	FAMILY NAME	FIRST GIVEN NAME	SECOND GIVEN NAME
	RESIDENCE & CITIZENSHIP	CITY	STATE OR FOREIGN COUNTRY	COUNTRY OF CITIZENSHIP
	POST OFFICE ADDRESS	POST OFFICE ADDRESS	CITY	STATE & ZIP CODE/COUNTRY
<p>I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.</p>				
SIGNATURE OF INVENTOR 204		SIGNATURE OF INVENTOR 205		SIGNATURE OF INVENTOR 206
DATE		DATE		DATE

MF:FORMS/COMDEC.WP 7/85